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Interplay between synaptic GPCRs in Alzheimer's Disease

Interplay between synaptic GPCRs in Alzheimer's Disease

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Abstract

Despite Alzheimer's disease (AD) being the most common form of dementia, our understanding of the molecular mechanisms that underlie the severe cognitive impairments of AD patients remains very incomplete. A well-supported hypothesis is that AD pathology disrupts neuronal communication at the synapse, a process thought to be crucial for memory function. Amyloid beta (Aβ) and phosphorylated tau (P-Tau) are key molecules in driving the impairment of synaptic function, but the molecular mechanisms involved in their neurotoxic effects are not fully characterised. Compelling evidence has positioned two types of synaptic G-protein coupled receptor (GPCRs), muscarinic acetylcholine receptors (mAChRs) and metabotropic glutamate receptors (mGluRs), as emerging mediators of such effects. They are both coupled to the same intracellular pathways but still appear to play differential roles in pathological mechanisms. Research has shown that blockade of mGluRs and upregulation of mAChRs are beneficial for synaptic function in *in vitro* and *in vivo* models of AD. The aim of this study is first to investigate the consequences of mGluRs activation in physiological conditions, to assess whether it is sufficient to cause synaptic impairments and whether it can regulate mAChRs function. Second, to characterise the expression of these synaptic GPCRs in the pathology of AD, to evaluate whether their potential interplay may be relevant to disease mechanisms. Third, to investigate the effects of P-tau, a key mediator of AD pathology, on the function of these synaptic GPCRs. Results from this study showed a functional interplay between mGluRs and mAChRs and a differential regulation of both their protein levels in AD brains and their function in the presence of P-tau. Altogether, this work provides new insights into possible mechanisms that contribute to synaptic dysfunction in AD, which will help to design therapeutic strategies to combat this devastating disorder.

Acknowledgements

I am now certain that doing a PhD is not an easy endeavour. Although I consider I have done my best to accomplish what it was required of me, I am well aware that this thesis is the result not only of my hard work but the support of many who helped me during this journey.

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When I started this project, I was told something that I think it summarises the challenges and satisfactions of doing a PhD, and that is: “No pain, no gain”. I therefore dedicate this thesis to everyone who, in one way or another, has contributed in turning the “pain” into a “gain”.

Thanks to you all,

Celia

Author’s declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

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Abbreviations

Aβ	Amyloid β-peptide
Aβo	Amyloid β oligomers
ACh	Acetylcholine
aCSF	Artificial cerebrospinal fluid
AD	Alzheimer's disease
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
APP	Amyloid precursor protein
CCh	Carbachol
DAG	Diacylglycerol
fEPSP	Field excitatory postsynaptic potential
GPCR	G-protein coupled receptor
IP_3	Inositol 1,4,5-trisphosphate
LTD	Long-term depression
LTP	Long-term potentiation
mAChR	Muscarinic acetylcholine receptor
mGluR	Metabotropic glutamate receptor
NFTs	Neurofibrillary tangles
NMDAR	N-Methyl-D-aspartic acid receptor
P-hTau	Phosphorylated human tau
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKC	Protein kinase C
PLC	Phospholipase C
PSD	Postsynaptic density

Chapter 1 Introduction

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1.1 Alzheimer's disease is a memory disorder

1.1.1 Dementia

Alzheimer's disease (AD) is classified as a neurodegenerative disorder and constitutes the most common cause of dementia (Alzheimer's Association 2018). The word dementia does not refer to a specific disease but rather is an overall term that refers to a set of symptoms that affect cognitive function, including memory, thinking, behaviour and emotions, which characterize progressive neurodegenerative brain disorders (Scott & Barrett 2007). Apart from AD, other causes of dementia have been described such as vascular dementia, which may occur after stroke, dementia with Lewy Bodies (DLB), frontotemporal lobar degeneration (FTLD), Parkinson's disease (PD) dementia and Creutzfeldt-Jakob disease, among others (Pohjasvaara et al. 2000; Mrak et al. 2007; Hodges et al. 2004; Emre 2004; Dupiereux et al. 2009). Regardless of the cause, all patients with dementia are eventually unable to perform day-to-day activities and to take care of themselves, needing help with all aspects of daily life (Scott & Barrett 2007). This fact makes AD patients extremely dependent on their family and friends, having a dramatic impact on the life of both. Likewise, given the long duration of the disease, patients live in a state of disability for very long periods of time. The number of people living with dementia in the UK is forecast to be over 1 million by 2025 (Prince et al. 2014). Economically speaking, the average annual cost per patient is £32,250 in the UK, with a total cost to society of £26.3 billion (Prince et al. 2014). These data clearly indicate the huge socio-economic impact of dementia and therefore the necessity of searching for more efficient therapeutic interventions for AD, for which a cure has not been found yet.

1.1.2 Symptoms and diagnosis of AD

The first symptom in AD patients is the inability to remember new information due to the malfunction of neurons located in memory-related areas of the brain, such as the hippocampus. As the disease progresses and other brain areas degenerate, multiple cognitive difficulties arise, although the pace at which they develop varies from person to person (Huang & Mucke 2012). These include difficulties completing day-to-day activities, trouble solving problems, speaking and writing, changes in mood and confusion with time and place (Alzheimer's Association 2018). Although these are classic symptoms of AD patients, they may be caused by other dementias such as the previously mentioned ones (Scott & Barrett 2007;

Dupiereux et al. 2009). Therefore, one important challenge in AD is finding efficient diagnostic tools that allow for clear identification of the disease and consequently appropriate treatment.

One of the main problems in the diagnosis and treatment of AD is that when the above symptoms develop, the pathophysiological changes in the brain are already in a very advanced state. Usually patients with AD are diagnosed at late stages when the diagnosis is fundamentally of clinical nature, through cognitive tests and neuropsychological evaluation (McKhann et al. 2011). Importantly, AD-related brain alterations may start 20 years before the emergence of memory loss and other noticeable symptoms (Sperling et al. 2011; Price & Morris 1999). For this reason, it is widely believed that future treatments to stop or slow down the progression of the disease (disease-modifying treatments) should be administered during the preclinical stage to have the most beneficial effects (Korolev 2014). The difficulty remains, however, in detecting AD at early stages. One promising approach is the identification of biomarkers that define the preclinical (asymptomatic) stage of AD and that can be used at the clinic. For example, a biomarker model has been proposed based on the detection of the two of the pathological hallmark proteins in AD, namely amyloid β42 (Aβ42) and tau protein, in cerebrospinal fluid (CSF) (Jack et al. 2010; Sperling et al. 2011; Tarawneh et al. 2015). In addition, imaging techniques, such as positron emission tomography (PET), to track levels of amyloid protein and magnetic resonance imaging (MRI), to detect brain atrophy that correlates with cognitive decline, have been tested to detect early AD in clinical trials (Hampel et al. 2010; Frisoni et al. 2010; Putcha et al. 2011; Albert et al. 2011; McKhann et al. 2011). These studies have shown that although the definitive biomarker profile that defines preclinical AD is still difficult to identify, moving towards early intervention is a promising avenue to prevent AD.

1.1.3 Aetiology and risk factors of AD

As with many other chronic diseases, there is no single cause for AD but rather it is the result of complex interactions between multiple factors. These include genetic, epigenetic and environmental factors that act in combination to cause aberrant brain function and increase the likelihood of developing the disease (Huang & Mucke 2012). The only exception of this is the familial Alzheimer's Disease (FAD) variant, which is caused by specific genetic mutations. So far, three genes for which mutated forms originate autosomal dominant forms of FAD have

been identified: APP, which codes for the amyloid precursor protein (APP), and presenilin 1 and 2 (PSEN1 and PSEN2), which code for the APP processing proteins (Bertram et al. 2010). The carriers of any of these mutations will develop FAD, which is characterized by an early-onset (<60 years old) and accounts for approximately less than 1% of AD cases (Bekris et al. 2010). The rest of the AD cases correspond to the sporadic AD variant (late-onset, >65 years old) where the aetiology is not that well established. However, various risk factors have been described in the literature, of which the greatest is age. Most of the AD cases are diagnosed at the age of 65 or older where differentiating between aging-related cognitive decline and AD is problematic (Craik 1994; Swerdlow 2007). From a clinical perspective, cognitive decline in the elderly must impede the subject's independence to perform daily activities to be considered pathological (Swerdlow 2011). From a neuropathological perspective, atrophy and gross changes in brain anatomy are features of both aging and AD, but they seem to follow distinct patterns. For instance, reductions in hippocampal volume and in specific populations within hippocampal neurons are more predominant in AD brains than in non-demented subjects (West et al. 1994; Frisoni et al. 2010). These studies have supported that although a significant degree of overlap in the manifestation of aging and AD-associated changes exits, AD is not part of the normal aging process (Swerdlow 2011). Importantly, age alone is not sufficient to cause AD, further supporting the multifactorial basis of AD aetiology (Sperling et al. 2011; Swerdlow 2007).

Genetics also plays an important role in the risk of having late-onset AD. There are various genes associated with the risk of developing the disease but the most robust one is the gene APOE. Of the three isoforms of this gene, namely, E2, E3 and E4, the E4 allele is considered the major genetic risk factor for AD (Corder et al. 1993). People with two E4 alleles are at a higher risk of developing AD than those with only one copy of the allele (Holtzman et al. 2012). There is also potential risk of AD associated with having mild cognitive impairment (MCI), traumatic brain injury (TBI), a family history of AD and cardiovascular risk factors, such as smoking and diabetes (Wei et al. 2016; Lye & Shores 2000; Loy et al. 2014; Green et al. 2002; Anstey et al. 2007; Gudala et al. 2013). Importantly, it seems that not only biological factors but also social factors could influence the likelihood of developing AD. Research is now pointing towards education and remaining socially and mentally active as means to reduce the risk of AD (Sando et al. 2008; Stern 2012).

In summary, AD probably develops as a consequence of an accumulation of risk factors that contribute to triggering neurodegenerative processes in the brain. Clinical examination significantly helps to identify these risk factors and in the case of aging, to differentiate it from AD. In addition, the assessment of neuropathological changes in demented brains is essential for the diagnosis of AD and has been largely associated to its aetiology, as they represent a clear sign of the disease.

1.1.4 Neuropathology of AD

At the macroscopic level, brains from patients with AD show a modest cortical atrophy, especially in the frontotemporal association cortex (Frisoni et al. 2010; Tarawneh et al. 2015; Gearing et al. 1995). However, it is only upon microscopic analysis of the *post-mortem* brain when a definitive diagnosis can be made. At this level, AD is characterized by three pathological hallmarks: amyloid plaques, neurofibrillary tangles and neuronal death. These cause serious brain damage, giving rise to structural and functional disruption of brain circuits (Perl 2010).

Neurofibrillary tangles

Neurofibrillary tangles (NFTs) are intracellular fibrous aggregations of hyperphosphorylated tau, a microtubule-associated protein and the primary constituent of the NFTs (Iqbal et al. 1989; Lee et al. 1989). Microtubules are dynamic protein structures that are part of the cytoskeleton of the cell. Through constant assembly and disassembly, microtubules function to maintain cell morphology by providing structural support and they are also involved in the transport of organelles in the cytosol and the separation of chromosomes during mitosis (Song & Brady 2015). The role of tau is to bind and stabilize microtubules and this function is determined by the phosphorylation state of tau (Lindwall & Cole 1984; Ando et al. 2016). Thus, non-phosphorylated forms of tau bind to microtubules whereas phosphorylation causes the dissociation of tau and microtubules tend to disassemble (Biernat et al. 1993; Lindwall & Cole 1984). Under pathological conditions, like AD, tau is hyperphosphorylated so the equilibrium of tau binding to the microtubules is altered (Iqbal et al. 1986; Grundke-Iqbal, Iqbal, Tung, et al. 1986; Goedert et al. 1992), having two important consequences. First, microtubules get unstable, which disrupts the characteristic neuronal morphology (typically very asymmetrical, with processes extending over

long distances) and the trafficking of cellular components along the axons. This greatly affects the functionality of the neurons and their highly elongated processes (Ballatore et al. 2007). Second, the levels of free (unbound) hyperphosphorylated tau increase in the cytosol, leading to its abnormal aggregation in fibrillary structures termed paired helical filaments (PHFs) (Iqbal et al. 1989). The ultrastructural analysis of these aggregates has shown that they are formed by pairs of fibrils that are bound in a helical fashion, hence the name. PHFs then self-assembly to form the filamentous inclusions called NFTs (A. del C. Alonso et al. 2001) **(Figure 1-1A).**

In a *post-mortem* brain, NFTs are observed with silver impregnation staining techniques or immunohistochemical approaches (Perl 2010). It is important to mention that, although NFTs are a cardinal neuropathological characteristic of AD, many other diseases display this lesion. The collectively named “tauopathies” are disorders defined by the aggregation of insoluble tau in the brain that most commonly cause dementia or degeneration of the motor system (Williams 2006). These include AD, which is the most common one, type C Niemann-Pick disease, post-encephalitic parkinsonism, corticobasal degeneration and Parkinson’s disease complex of Guam (Williams 2006). All these have in common the pathological intracellular accumulation of tau, which in turn leads, at least in part, to neurodegeneration and cell death (Regan et al. 2017). Therefore, therapies focused on decreasing the aggregation of tau or increasing microtubule stability are being investigated as potential tools to battle the so-called tauopathies (Ballatore et al. 2007; Anand et al. 2014).

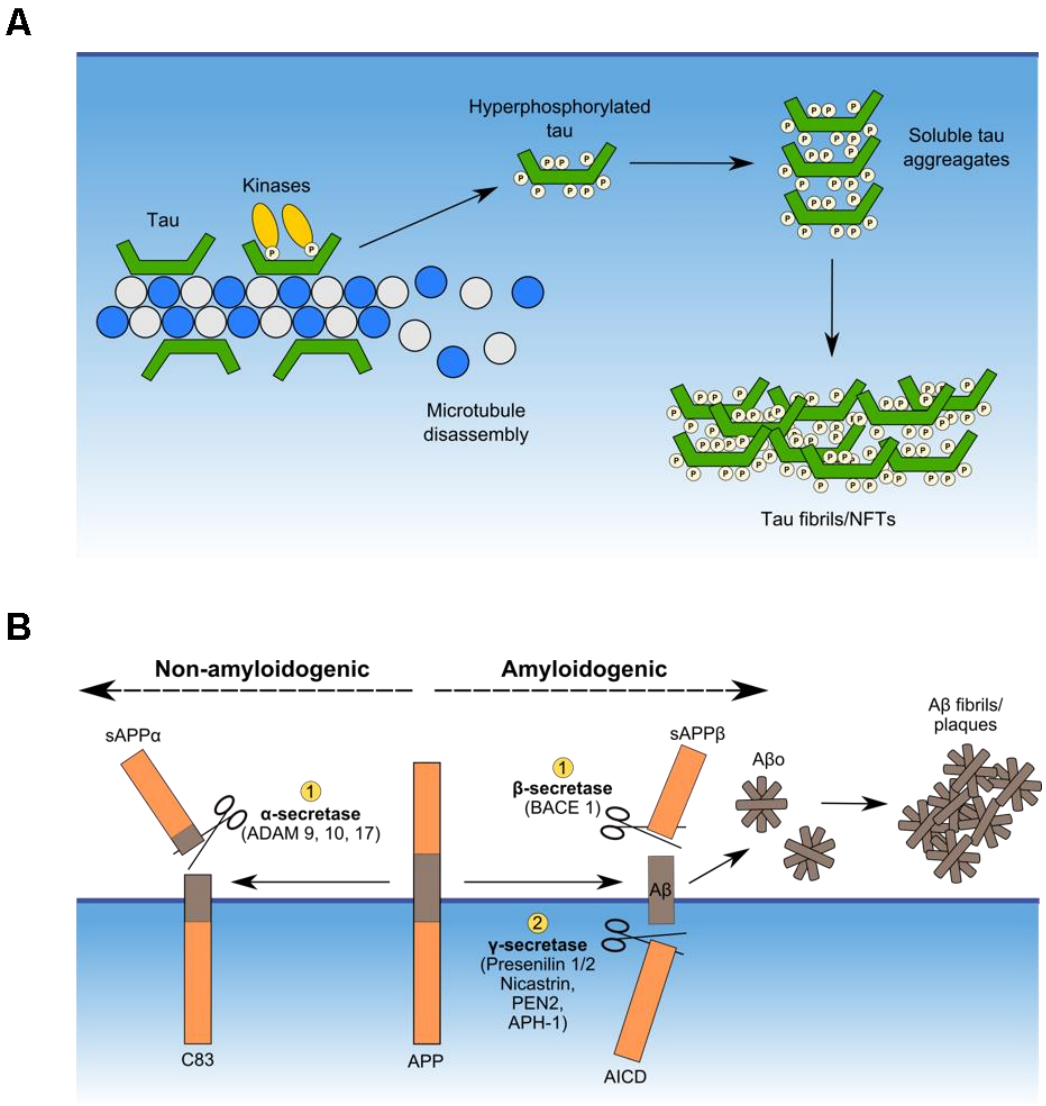


Figure 1-1. Key neuropathological processes in Alzheimer's disease. (A) Neurofibrillary tangles (NFTs) formation. In the intracellular space, hyperphosphorylated tau dissociates from microtubules causing them to depolimeryse while tau undergoes various aggregation states, culminating in the formation of insoluble fibrils. **(B) Amyloid precursor protein (APP) processing.** In the amyloidogenic pathway, sequential cleavage of APP by β- and γ-secretases results in the generation of Aβ peptides which first aggregate into soluble Aβ oligomers (Aβo) in the extracellular space and eventually into fibrils and plaques. Adapted from (LaFerla et al. 2007; Götz & Ittner 2008).

Amyloid plaques

The main constituent of amyloid plaques, also called “senile” or “neuritic” plaques, is the Aβ peptide that results of the proteolytic cleavage of a transmembrane glycoprotein termed APP protein (Kang et al. 1987). The precise role of the APP protein in the physiology of the neuron remains unknown, although there is evidence supporting its involvement in cell growth, motility and neurite outgrowth (Oh et al. 2009; Young-Pearse et al. 2007). The processing pathways that this

protein can undergo are well established, however **(Figure 1-1B)**. The non-amyloidogenic pathway consists in two enzymatic cleavages performed by α-secretase and then γ-secretase that do not generate pathological Aβ peptides; whereas in the amyloidogenic pathway the cleavage sequence changes to β-secretase and then γ-secretase, which produces the fragment of 4 kilo Daltons (kDa) known as Aβ peptide (O'Brien & Wong 2011). The Aβ peptide is released into the extracellular space where it aggregates, adopting a β-sheet configuration and, together with other proteins, forms the amyloid plaques (Palop & Mucke 2010; Castillo et al. 1997; Snow et al. 1996; Dickson et al. 1997).

Together with the genetic factor APO-E4, the accumulation of pathogenic Aβ aggregates in the brain greatly contributes to the risk of having AD and a decline in cognitive function (Farrer et al. 1997; Hardy & Selkoe 2002). One prominent hypothesis of how Aβ can impair cognition is based on the detrimental effects of Aβ on synaptic plasticity and signalling. A lot of evidence from animal models of AD supports the notion that Aβ reduces glutamatergic synaptic transmission (Hsia et al. 1999; Chapman et al. 1999; Walsh et al. 2002) and that a decrease in the number of surface α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPARs) and N-methyl-D-aspartate receptors (NMDARs) is a mechanism by which Aβ reduces excitatory synaptic transmission (Hsieh et al. 2006; Shankar et al. 2007). The current model of Aβ-mediated synaptic dysfunction relies on evidence showing that Aβ impairs long-term potentiation (LTP) (Cleary et al. 2005; Walsh et al. 2002) and enhances long-term depression (LTD) (J. H. Kim et al. 2001; Li et al. 2009; Hsieh et al. 2006) of synaptic transmission. Both LTP and LTD are considered two of the main molecular processes underlying memory function and its impairment is correlated to synaptic loss and memory problems in animal models (Martin et al. 2000). Importantly, growing evidence has shown that soluble Aβ oligomers play a more important role than Aβ fibrils and amyloid plaques in causing these effects (Shankar et al. 2008; W. L. Klein et al. 2001; Tomiyama et al. 2010; Cleary et al. 2005; Cheng et al. 2007; Selkoe 2008) and correlate better with cognitive decline (Mucke et al. 2000; Näslund et al. 2000; McLean et al. 1999). Therefore, efforts are focused in understanding the pathological mechanisms triggered by oligomeric species of Aβ.

1.1.5 Pharmacological strategies against AD

Despite these advances made in AD research, we are far from finding a drug treatment that stops the fatal consequences of the disease. To date, only six drugs are approved by US Food and Drug Administration (FDA) that can be divided in two categories: acetylcholine esterase inhibitors (AChEIs) and one NMDA receptor antagonist. The former includes the drugs rivastigmine (Exelon), galantamine (Razadyne), tacrine (Cognex), and donepezil (Aricept) (brand name is indicated between brackets). The latter is the drug memantine (Namenda), the only one that appears to be effective at later stages of the disease (Alzheimer's Disease International 2015). The sixth drug treatment has been approved relatively recently and combines donepezil and memantine (Alzheimer's Association 2018). AChEIs act to increase the levels of ACh in the brain and although they slow the progression of cognitive symptoms, they do not change the course of the disease and half of the people taking these drugs do not respond to them (Kumar & Singh 2015). For this reason, researchers are shifting their efforts to the search of therapies that can stop disease progression.

Due to the complex nature of AD pathology, our understanding of the molecular mechanisms that govern the disease is constantly changing. This represents a hurdle for a fast development of disease-modifying treatments, for which a better insight of the molecular pathways of the disease is needed (Anand et al. 2014). Even though, the therapeutic field in AD has grown in parallel to discoveries regarding the pathological mechanisms underlying different aspects of the disease. Thus, there is a wide range of strategies in therapeutic research in AD, as diverse as the mechanisms involved in the pathogenesis. Very broadly, these include modulating levels of various neurotransmitters and intracellular signalling cascades, amyloid (Citron 2010; Golde et al. 2011; De Strooper et al. 2010) and tau (Gura 2008; Schirmer et al. 2011; Morris et al. 2011) based therapies, oxidative stress reduction (Lee et al. 2010), mitochondrial targeted therapy (Bezprozvanny 2010) and anti-inflammatory therapy (Cole & Frautschy 2010), among others (Kumar & Singh 2015; Folch et al. 2016). These studies show that it is without question that a better insight of the molecular pathways of the disease is needed in order to develop efficacious treatments (Anand et al. 2014).

1.2 Synaptic function in the hippocampus: relation to memory and AD

The discovery that electrical activity underpins nerve function dates back to 1766 when Luigi Galvani found that an electric current applied to a muscle could provoke its contraction. Almost two centuries later, Santiago Ramón y Cajal proposed that the nervous system was composed of billions of discrete nerve cells (Cajal 1894), for which he is still considered the father of modern neuroscience. Based on this concept, Wilhelm Waldeyer enunciated the "neuron doctrine" in 1891, where he named the separate entities that composed the nervous system "neurons" (von Waldeyer-Hartz 1891). This doctrine established the fundamental basis for understanding how information is transported and stored in the nervous system. In 1897, the term synapse was coined by Charles Sherrington to define the specializations found in neurons at which electrical and chemical signals are transmitted (Sherrington 1906). Therefore, at the end of the 19th century these discoveries lay the groundwork for our exponentially growing understanding of the electrical processes taking place in the nervous system. The next landmark discovery in this field was the "plastic" nature of synapses, both from anatomical and functional perspectives. This gave rise to the term "synaptic plasticity", introduced by Jerzy Konorski in 1948 to define activity-driven changes in synaptic efficacy that persisted in time, providing a mechanism of information storage in the brain (Konorski 1948). Shortly after, Donald Hebb formulated his famous postulate to explain the adaptation that neurons undergo during learning processes*: "When an axon of cell A is near enough to excite a cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased"* (Hebb 1949). This theory, known as Hebb's rule, is often summarised as "neurons that fire together wire together" and provides the first basic description of synaptic plasticity. Importantly, this set the stage to subsequent theories and models linking activity-dependent changes occurring at synapses to memory and learning processes. Currently, the study of synaptic function is considered the key to understand information processing in the brain and a great part of it is still based on Hebbian concepts (Brown & Milner 2003). In addition, it is now clear that synaptic dysfunction is one prominent pathological hallmark in AD and very likely plays an important role in the severe memory impairments at early stages of the disease (Selkoe 2002). Therefore, understanding how synapses function has become an essential requirement not only in the context of memory and learning

but also to elucidate the molecular basis of pathological mechanisms operating in AD.

1.2.1 Classification of memory

Memory can be defined as the process of encoding, storing, and retrieving information (Jahn 2013). According to how long the information is stored in the brain for, memory has been divided in three categories: immediate or working memory (lasts for seconds and allows for tasks in the immediate future), short-term memory (lasts for seconds to minutes) and long-term memory (lasts for days, weeks or a lifetime) (McGaugh 1966) **(Figure 1-2)**. Long-term memory is further subdivided according to the type of information that is stored. Thus, declarative (or explicit) memory refers to the memory of facts (semantic memory) and events (episodic memory), that requires conscious recall of factual information. In contrast, non-declarative (or implicit) memory refers to unconscious recalling of memories to perform a task, such as skill learning (Kandel et al. 2013). In AD patients, memory loss is one of the major and primary symptoms, particularly deficits in episodic memory.

The medial temporal lobe (MTL), where the hippocampus is located, is widely accepted to be a central region for memory function (Tulving & Markowitsch 1998). For this reason, the study of the physiological processes underlying memory has been focused on this brain region. Indeed, a vast amount of evidence arising from studies in the hippocampus has led to the discovery of synaptic plasticity, which is now believed to be the underpinning physiological mechanism for learning and memory (Martin et al. 2000).

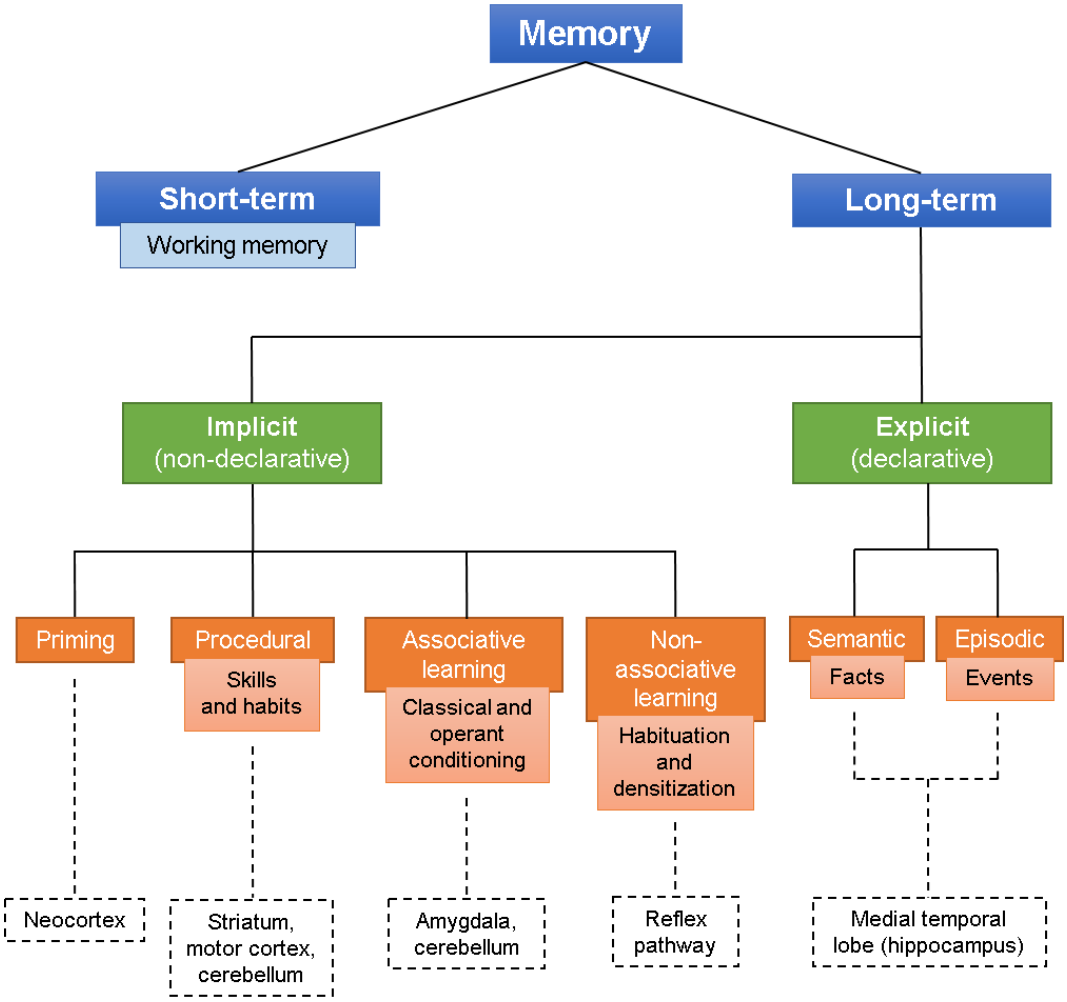


Figure 1-2. Taxonomy of memory systems. Memory is classified into different "systems" according to the time information is stored for and the type of information stored. The interdependence of these systems is not fully delineated but it is clear that each system is mediated by distinct brain regions. Adapted from (Kandel et al. 2013; Squire 2004).

1.2.2 Synaptic transmission

Synapses are the basic functional units of the nervous system where information is conveyed from one neuron to another. Depending on the modality in which synaptic transmission occurs, synapses can be classified in two categories: electrical and chemical (Pereda 2014). In electrical synapses, pre- and postsynaptic intracellular spaces are connected by ion channels termed gap junctions, that allow a bidirectional flow of ions between two adjacent neurons. Synaptic transmission in chemical synapses occurs from the presynaptic terminal to the postsynaptic terminal, by means of a chemical molecule termed neurotransmitter. The presynaptic terminal is characterised by the presence of synaptic vesicles in a region termed the active zone, each vesicle containing thousands of molecules of neurotransmitter (Südhof 2012b; Harris & Sultan 1995)

(**Figure 1-3**). Upon arrival of an action potential and depolarization of the presynaptic terminal, voltage-gated calcium channels (VGCCs) are activated and calcium concertation rises in the intracellular space. This causes vesicles to fuse with the presynaptic membrane in a calcium-dependent manner, resulting in the neurotransmitter being released into the space that separates both terminals, the synaptic cleft (Katz & Miledi 1965). Neurotransmitter molecules then bind to receptors located in the postsynaptic terminal. Specifically, they bind to a region with a high density of postsynaptic receptors that is termed postsynaptic density (PSD) and it is a highly specialised structure (Kennedy 1997). Importantly, the composition and architecture of this structure has functional implications. Synapses with a thick PSD are known as asymmetrical or type I synapses and have been shown to be excitatory (Gray 1959; Andersen et al. 2006). Symmetrical or type II synapses usually lack a PSD and are thought to be inhibitory (Gray 1959). The binding of neurotransmitter to its specific receptor triggers the activation of the receptor. In the case of G protein-coupled receptors (GPCR), binding of the neurotransmitter results in the activation of the coupled G protein, triggering second messenger signalling pathways that can result in depolarization or hyperpolarization of the membrane (Betke et al. 2012). In the case of ligand-gated ion channel receptors, the channel opens in response to neurotransmitter binding and ions flow in or out of the intracellular space. For instance, glutamate, the major excitatory neurotransmitter, causes the opening of glutamate receptors which are ion channels permeable to sodium and calcium. This ion flow constitutes the excitatory postsynaptic current (EPSC) and causes a small depolarization of the membrane, giving rise to an excitatory postsynaptic potential (EPSP). In the case of inhibitory postsynaptic currents (IPSC), such as chloride currents mediated by γ-aminobutyric acid A ($GABA_A$) receptors, hyperpolarization of the membrane occurs instead, and the associated response is hence termed inhibitory postsynaptic potential (IPSP). The sum of multiple EPSPs can cause the activation of regenerative currents that in turn trigger the generation of an action potential. Conversely, IPSPs drive the membrane away from the threshold of action potential firing, therefore causing synaptic inhibition of the postsynaptic terminal.

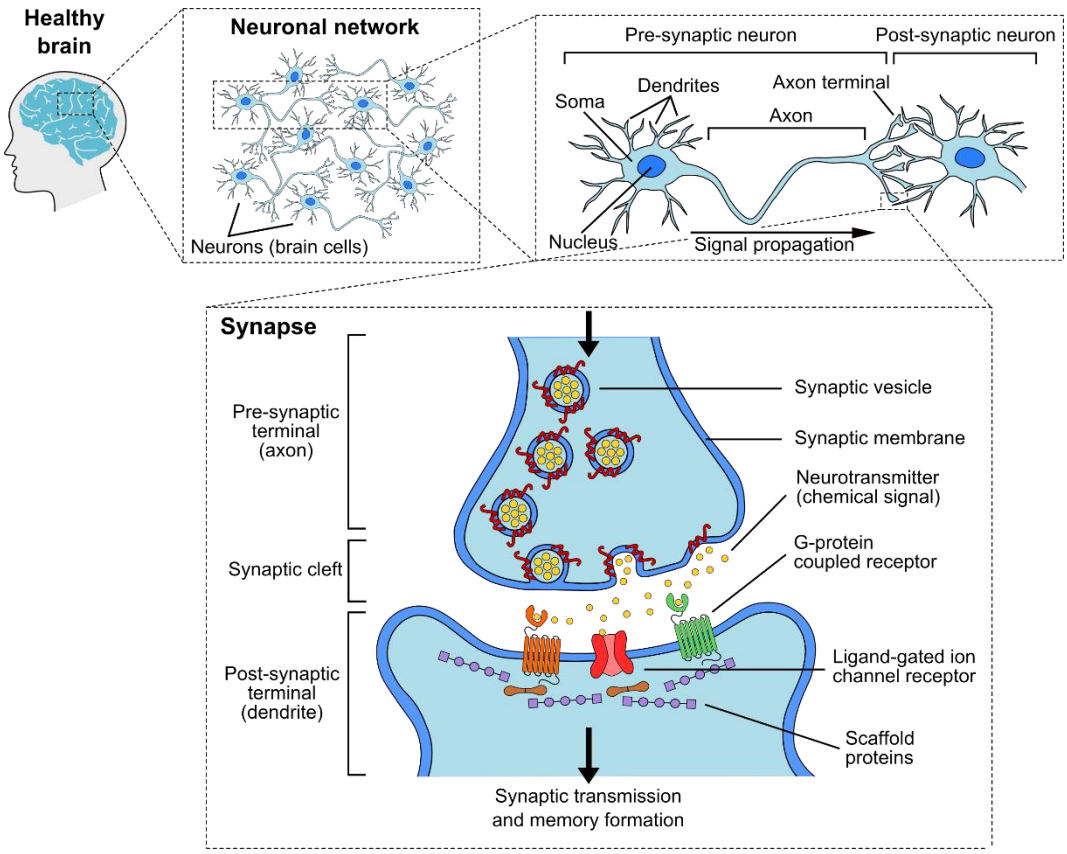


Figure 1-3. Synaptic transmission in healthy brain. In a healthy brain, synaptic transmission occurs normally: upon receiving a stimulus, neurotransmitter is released from the presynaptic terminal into the synaptic cleft where it binds to postsynaptic receptors located on the postsynaptic membrane. Their activation by neurotransmitter molecules results in the propagation of the stimulus in the postsynaptic terminal and normal synaptic function.

Synaptic transmission is the basis for information flow and storage in the brain and ultimately memory processes. Therefore, impairments in synaptic transmission may lead to deficiencies in synaptic function which are thought to underlie cognitive problems such as memory loss. Indeed, in pathological conditions such as AD, the presence of pathological molecules, mainly Aβ and hyperphosphorylated tau, triggers a series of events that culminate in disrupted synaptic transmission and neurodegeneration (**Figure 1-4**).

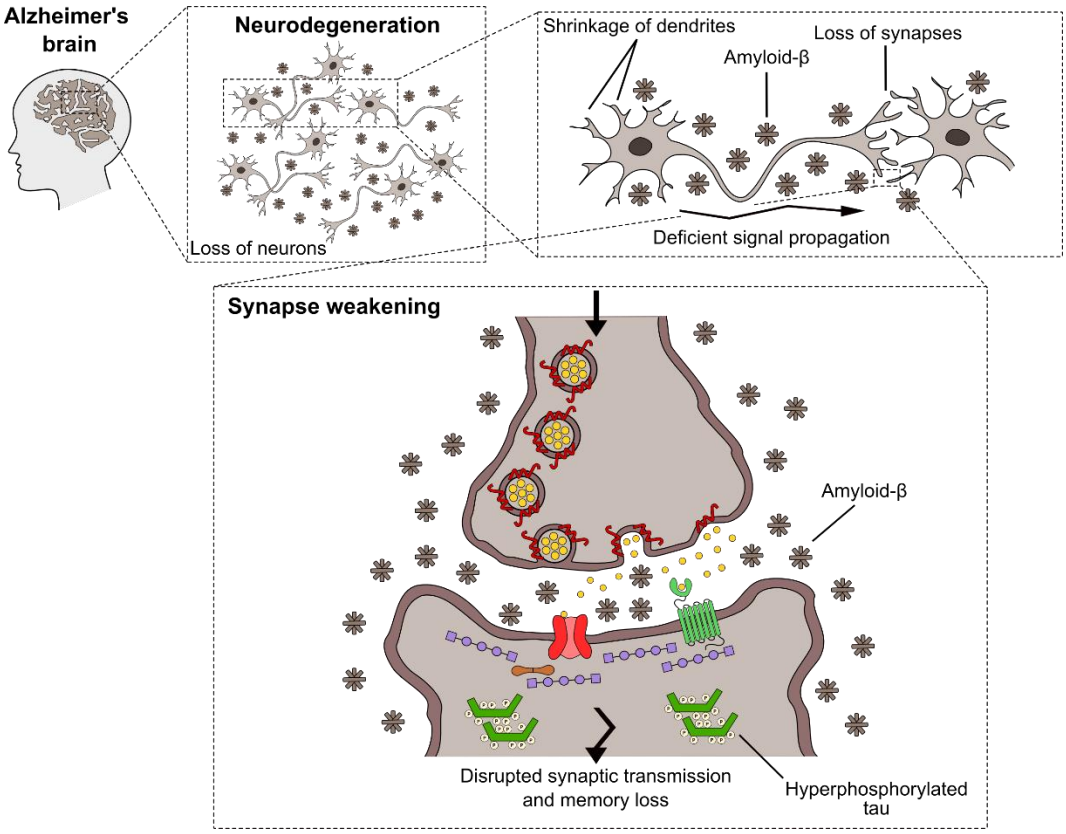


Figure 1-4. Synaptic transmission in neurodegenerative brain. In neurodegenerative conditions like in Alzheimer's disease, synapses weaken due to the presence of pathological molecules resulting in impaired synaptic transmission. This may underlie the memory loss associated with the disease.

In summary, neurotransmitter release from the presynaptic terminal generally results in a change in the excitability of the postsynaptic membrane. The direction and magnitude of this change depends on various factors such as the amount of neurotransmitter released, the initial voltage of the membrane, the spatial distribution of the synapse and the density and distribution of receptors present in the postsynaptic membrane. Out of these factors, synaptic receptors are key modulators of synaptic transmission and, therefore, understanding their malfunction in disease is vital for successful therapeutic interventions.

1.2.3 Synaptic receptors

Glutamatergic receptors

As mentioned previously, glutamate is the main excitatory neurotransmitter in the brain, acting through two major types of receptors, ionotropic and metabotropic. Ionotropic glutamate receptors are heteromultimeric ligand-gated ion channels, further subdivided in three major classes according to their pharmacological

properties: AMPA, NMDA and kainate receptors. Metabotropic glutamate receptors are GPCRs further subdivided according to their sequence similarity, signal transduction mechanisms and pharmacological interactions into groups I, II and II **(Table 1).**

Table 1. Synaptic receptors classification and mechanisms of action.

Neurotransmitter	Type of receptor	Receptor name	Mechanism of action
Glutamate	Ionotropic	NMDAR	Sodium, potassium and calcium ion channel
		AMPAR	Sodium, potassium and calcium (GluA2-lacking) ion channel
		Kainate	Sodium and potassium ion channel
	Metabotropic	Group I mGluR1 mGluR5	GPCR coupled to $G_{q/11}$ and PLC activation
		Group II mGluR2 mGluR3	GPCR coupled to $G_{i/o}$ and AC inhibition
		Group III mGluR4 mGluR6 mGluR7 mGluR8	
Acetylcholine	Nicotinic	nAChRs	Cation ion channels
	Muscarinic	mAChR1 mAChR3 mAChR5	GPCR coupled to $G_{q/11}$ and PLC activation
		mAChR2 mAChR4	GPCR coupled to $G_{i/o}$ and AC inhibition
GABA	Ionotropic	$GABA_A$ $GABA_C$	Chloride ion channel
	Metabotropic	$GABA_B$	GPCR coupled to $G_{i/o}$ and AC inhibition

PLC: Phospholipase C, AC: Adenylyl cyclase.

AMPA receptors. They are ligand-gated ion channels activated by the agonist AMPA and composed of different combinations of four subunits (GluA1-4) (Keinänen et al. 1990; Hollmann & Heinemann 1994). Subunit composition is very important since it determines the electrophysiological properties of the channel. For instance, GluA2-containing AMPARs display very low permeability for calcium (Sommer et al. 1991). However, all AMPARs are permeable to sodium and potassium and display very fast binding kinetics and high opening probability (Jonas et al. 1993). They also deactivate rapidly when glutamate is cleared from the synaptic cleft (Colquhoun et al. 1992), or if glutamate is not cleared, they desensitise quickly and recover slowly (Mosbacher et al. 1994). These biophysical

properties make AMPARs the main mediators of fast excitatory transmission, a role that has been deeply explored in the hippocampus where they are expressed at almost all excitatory synapses (Wenthold et al. 1996).

Kainate receptors. These receptors are composed of five subunits (GluK1-5) (Hollmann & Heinemann 1994). They share similar biophysical properties with AMPAR, such as rapid opening and desensitisation kinetics and calcium permeability depending on subunit composition (Bowie & Mayer 1995; Kamboj et al. 1995). They are also expressed in the hippocampus although their contribution to synaptic function is less well understood than that of the other glutamate receptors (Cossart et al. 2002). However, evidence has emerged for a role of kainate receptors in controlling neurotransmitter release in the presynaptic terminal (Rodríguez-Moreno et al. 1997; Vignes et al. 1998), axon excitability (Semyanov & Kullmann 2001) and synaptic plasticity (Contractor et al. 2000).

NMDA receptors. They are heteromultimers composed of four subunits derived from three families: GluN1, GluN2A-2D and GluN3 (McBain & Mayer 1994; Nakanishi 1992). On the one hand, the GluN1 subunit binds the co-agonist glycine, which is essential for NMDAR to function (Kuryatov et al. 1994). On the other hand, GluN2A-2D subunits contain the glutamate-binding site and they are differentially expressed across the brain (Laube et al. 1997). The GluN3 subunit is not well characterised and seems to not play a role in the hippocampus (Chatterton et al. 2002). Having a second agonist-binding site is one well-characterised feature of the NMDAR, since both glycine and glutamate binding are necessary for activation of the receptor (Johnson & Ascher 1987). Another particular feature of these receptors is that in addition to their permeability to sodium and potassium, they have high permeability to calcium and monovalent cations, which is intimately related to their pivotal role in long-term synaptic plasticity (Ascher & Nowak 1988). The third important characteristic of NMDARs is that they only activate, and therefore mediate ion flux and EPSCs, when the membrane is depolarised. This is because at resting membrane potential, magnesium ions are blocking the ionophore of the receptors and impeding ion flux through it, even if glutamate and glycine are bound to the receptor (Mayer et al. 1984; Nowak et al. 1984). At depolarised potentials, this blockade is removed and the receptor can activate.

Both AMPARs and NMDARs are expressed at much higher density at synapses than at extrasynaptic membranes (Bekkers & Stevens 1989). As a consequence

of their kinetic properties, AMPARs mediate the fast component of the EPSC at negative potentials (Kleppe & Robinson 1999; Koike-Tani et al. 2005) whereas NMDARs account for the slow component of the EPCS that emerges at depolarised potentials (Forsythe & Westbrook 1988; Umemiya et al. 1999). These receptors have become central to the study of brain function since activation of both receptors provides the molecular substrate for synaptic plasticity mechanisms occurring in the brain (Lissin et al. 1998; Rong et al. 2001; Shi et al. 1999; Malenka 2003).

Metabotropic glutamate receptors (mGluRs). These are GPCRs and as such, they indirectly alter membrane excitability by the transduction of extracellular signals (i.e. glutamate) to intracellular signalling cascades. Molecular cloning has revealed eight members of mGluRs which have been subdivided into three groups according to their signal transduction mechanisms, sequence similarity and pharmacological properties (Conn & Pin 1997). Group I (mGluR1 and mGluR5) are coupled to phosphoinositide hydrolysis and are selectively activated by 3,5-dihydroxyphenylglycine (3,5- DHPG); group II (mGluR2 and mGluR3) and group III (mGluR4, mGluR6, mGluR7 and mGluR8), both negatively coupled to adenylate cyclase, have different selective agonists, LY379268 for group II and 2-amino-4-phosphonobutyrate for group III (Ferraguti & Shigemoto 2006; Wisniewski & Car 2002; Schoepp et al. 1999). Being type C GPCRs, mGluRs display the structural characteristics of this superfamily: a seven transmembrane domain (7TM), an extracellular amino-terminal domain, where the glutamate binding pocket is located, and a cytoplasmic carboxy-tail that mediates the activation of the coupled G-protein and the transduction of intracellular signalling cascades (Niswender & Conn 2010; Pin & Duvoisin 1995; Rosenbaum et al. 2009). The 7TM domain plays an important role in the regulation of receptor activity because it contains the binding sites for allosteric modulators (Brauner-Osborne et al. 2007). The previously described structure applies to an mGluR monomer but it has been demonstrated that dimerization is required for agonist activation of mGluRs (El Moustaine et al. 2012; Rondard & Pin 2015) although a single 7TM domain is responsible for G-protein activation *per se* (Hlavackova et al. 2005; Goudet et al. 2005). For instance, an mGluR5 homodimer is formed by two monomers covalently linked by disulphide bridges between conserved cysteines in the amino terminal domain (Romano, Yang, et al. 1996).

The synaptic location of these receptors also varies with different groups. Thus, group I mGluRs are predominantly found in postsynaptic elements, group II are expressed at both pre- and postsynaptic sites and group III are primarily expressed at presynaptic terminals (Baude et al. 1993; Shigemoto et al. 1993; Lujan et al. 1996). Within the postsynaptic element, group I mGluRs are distributed perysynaptically, where they are thought to be involved in detecting glutamate present for long periods of time (Baude et al. 1993) and in mGluR-dependent forms of synaptic plasticity (Grover 1998; Balschun et al. 1999; Wu et al. 2004; Collingridge et al. 2010; Jo et al. 2010). Similarly, group II mGluRs locate to the extrasynaptic site of the presynaptic terminal where they act as auto-receptors that regulate neurotransmitter release in response to the levels of glutamate built-up (Scanziani et al. 1997). Group III mGluRs have been shown to be present at GABAergic synapses and may have a similar role than group II (Shigemoto et al. 1997; Semyanov & Kullmann 2000).

Cholinergic receptors (AChRs). Cholinergic receptors are divided into ionotropic (nicotinic) and metabotropic (muscarinic) receptors. Nicotinic receptors are pentameric units and have been shown to enhance glutamate evoked GABA release (McGehee et al. 1995). Muscarinic receptors are GPCRs divided into five types: mAChR1, mAChR3 and mAChR5 couple to $G_{q/11}$ and the activation of PLC whereas mAChR2 and mAChR4 couple to $G_{i/o}$ and adenylyl cyclase activation (Caulfield & Birdsall 1998). Muscarinic receptors play a role in the regulation of synaptic transmission. For example, their activation can influence action potential properties (Figenschou et al. 1996). Furthermore, activation of mAChR1 results in the potentiation of NMDAR-mediated depolarisations in the hippocampus (Markram & Segal 1992). In regions such as the perirhinal cortex, mAChRs have a central role in synaptic plasticity where they mediate a form of LTD that is thought to be the molecular substrate for visual recognition memory (Massey et al. 2001).

GABAergic receptors. γ-aminobutyric acid (GABA) is the major inhibitory neurotransmitter and therefore GABA receptors mediate inhibitory synaptic transmission. They are classified into ionotropic $GABA_A$ receptors and metabotropic $GABA_B$ receptors. Some include another type of ionotropic $GABA_C$ receptors but this is considered by many as a variant of $GABA_A$ (Barnard et al. 1998). $GABA_A$ and $GABA_C$ receptors are widely expressed in the hippocampus

where they contribute to different types of inhibition on excitatory synapses (Overstreet & Westbrook 2001; Andrade et al. 1986; Misgeld et al. 1995).

1.2.4 Synaptic plasticity

The concept of synaptic plasticity is defined as the ability of a synapse to strengthen or weaken over time in response to increases or decreases in its activity (Martin et al. 2000). The idea of a change in the "strength" of synapses was first proposed by Donald Hebb in 1949 (Morris 1999). Currently, this plasticity of synapses is widely believed to be crucial for the long-lasting experience-dependent changes in neuronal function occurring during learning and memory processes (Martin et al. 2000). As mentioned previously, the best characterized forms of synaptic plasticity are LTP and LTD (Bliss & Collingridge 1993; Collingridge et al. 2010). In LTD and LTP, the efficiency of transmission at a synapse is respectively decreased or increased as a consequence of repeated activity at that same synapse (Bliss & Collingridge 1993; Martin et al. 2000; Collingridge et al. 2010; Voglis & Tavernarakis 2006). The existence of LTP was first demonstrated by Terje Lømo and Tim Bliss in 1973 (Lømo 1966; Bliss et al. 1973) followed a few years later by the discovery of LTD (Lynch et al. 1977), both in the hippocampal formation. Since then, these two processes have become an essential part of our understanding of learning and memory mechanisms.

Synaptic plasticity is governed by numerous factors but of particular importance is the regulation of synaptic receptors located at the postsynaptic membrane. Specifically, excitatory synaptic transmission greatly depends on the number of NMDARs and AMPARs present at the postsynaptic terminal (Malenka 2003; Bekkers & Stevens 1989). The best example of this is the NMDAR-dependent LTP (NMDAR-LTP) that occurs at excitatory synapses at the CA1 region of the hippocampus, among other regions. This form of LTP has been extensively studied and a great amount of our current knowledge in the field of synaptic plasticity comes from the molecular mechanisms described in the hippocampus (Citri & Malenka 2008).

The activation of NMDARs can result in LTP or LTD, depending to a great extent on the intracellular calcium levels reached during the process (Lynch et al. 1983; Malenka et al. 1988; Mulkey & Malenka 1992; Cummings et al. 1996). Generally speaking, high levels of intracellular calcium and activation of NMDARs result in

the insertion of AMPARs in the synaptic membrane and LTP (Malenka 2003; Malenka 1991; Lu et al. 2001; Hayashi et al. 2000; Shi et al. 1999). This process is divided in two broad stages, early-phase LTP (E-LTP), where LTP is induced, and late-phase LTP (L-LTP), where maintenance mechanisms are activated. During E-LTP, glutamate released from the presynaptic terminal activates glutamate receptors located in the postsynaptic membrane, which generates a flux of sodium going inside the neuron. This causes the depolarization of the postsynaptic terminal and, when a threshold level is reached, the magnesium block in the NMDARs inophore is released, allowing an influx of calcium (Bliss & Collingridge 1993; Mayer et al. 1984). The increase in the intracellular calcium concentration then results in the activation of various signalling cascades, including activation of kinases, that lead to the insertion of AMPARs in the postsynaptic membrane and LTP (Lu et al. 2001; Pickard et al. 2001; Shi et al. 1999; Hayashi et al. 2000). Unlike E-LTP, L-LTP requires *de novo* protein synthesis (Stanton & Sarvey 1984) that is necessary for the morphological changes that allow growth of new neuronal connections and a long-lasting expression of LTP (Yuste & Bonhoeffer 2001; Desmond & Levy 1986; Stewart et al. 2000; Toni et al. 1999; Weeks et al. 2001). It should be noted that apart from NMDAR-dependent LTP, other forms of LTP have been described in various regions throughout the brain, for example LTP dependent on mGluRs activation (Grover 1998; Balschun et al. 1999; Wu et al. 2004).

In contrast to LTP, a modest increase in intracellular calcium levels and removal of AMPARs from the synaptic membrane results in LTD (Bliss & Collingridge 1993; Cummings et al. 1996; Carroll et al. 1999). Evidence has also supported that contrary to LTP, LTD is accompanied by a shrinkage in the size of dendritic spines (Nägerl et al. 2004; Zhou et al. 2004). Similarly to LTP, different forms of LTD that depend upon activation of non-NMDARs have been described in various brain regions (Collingridge et al. 2010; Jo et al. 2010).

Since the origins of synaptic plasticity, compelling evidence has shown that the co-existence of LTP and LTD at the synapses is the substrate for mechanisms that regulate the encoding and storing of memories (Takeuchi et al. 2014). Due to this, both forms of synaptic plasticity have been widely studied in the hippocampus, a brain region with a central role in memory processes (Bird & Burgess 2008) (Bird & Burgess 2008). Consequently, a very useful experimental approach to study LTP

and LTD has been the *in vitro* hippocampal slice preparation from rodent brain, that combined with electrophysiology techniques, has allowed for the investigation of the molecular mechanisms underlying learning and memory (Lein et al. 2011).

1.2.5 The hippocampal formation

The hippocampal formation, located in the medial temporal lobe of the brain, is one of the most studied structures in the mammalian brain in the memory field of modern neuroscience. There are two main reasons for this. First, there is compelling evidence that strongly points to the hippocampal formation as a brain region with a highly specific role in memory (Van Strien et al. 2009; Neves et al. 2008). Second, the performance of advanced techniques for the study of synaptic plasticity, such as electrophysiological recordings, is particularly convenient in this formation (Bortolotto et al. 2001). The reason being that the hippocampal formation is highly organised, with cells structured in a laminar distribution (Andersen et al. 1971; Witter et al. 2000). This allows for clear distinction and control over which are the projections being stimulated and which cell's electrical responses are being recorded (Andersen et al. 2006). Moreover, as outlined above, the hippocampus is one of the first regions affected by AD pathology (Braak et al. 2006), and therefore it is not only a suitable model to study the molecular correlates of memory but also their impairment in disease.

Anatomy of the hippocampal formation

The hippocampal formation refers to a group of adjoining regions in the medial temporal lobe (MTL) of the brain, characterised by a distinct cytoarchitecture (Squire & Zola-morgan 1991). These regions include the dentate gyrus, hippocampus, subiculum, pre-subiculum, para-subiculum and entorhinal cortex (Witter et al. 2000). As first described by Ramón y Cajal, an intrinsic unidirectional circuit of excitatory projections connects these regions (Ramón y Cajal 1893). Unlike many other brain regions, the hippocampal formation receives highly processed multimodal sensory information from the neocortex and thanks to its intrinsic network of neuronal connections it is capable of integrating and comparing this information (Burgess et al. 2002). Therefore, it is critical to understand the anatomy and connections of the hippocampal formation to understand its unique role in memory processing (Van Strien et al. 2009).

The entorhinal cortex, that receives information coming from sensory areas of the cortex, can be considered the first step in the hippocampal network. The entorhinal cortex sends projections, termed the perforant path, to the dentate gyrus, forming the major input pathway into the hippocampus (Witter & Amaral 1991; Steward & Scoville 1976). The hippocampus proper is the area containing the Cornu Ammonis (CA) which is subdivided in three regions in the rodent brain: CA1, CA2 and CA3 (Lorente de Nó 1934). Pyramidal cells are the main type of cells in the dentate gyrus and display a very characteristic structure, with basal and apical dendritic trees and one axon (Pyapali et al. 1998). These cells project their axons, termed mossy fibers, to the pyramidal cells in the CA3 of the hippocampus (Blackstad et al. 1970; Gaarskjaer 1978) (**Figure 1-5**). CA3 cells project their axons, termed Schaffer collateral, into the CA1 hippocampal region, that also contains pyramidal cells (Ishizuka et al. 1990). These CA1 cells then project to the subiculum (Amaral et al. 1991) and both CA1 cells and subiculum send projections to deep layers of the entorhinal cortex from where the processed information returns to the neocortex (Swanson & Cowan 1975). In the present study, electrophysiological recordings were obtained from the dendritic region of CA1 pyramidal cells (*stratum radiatum*) in field recording experiments or the soma of these cells (pyramidal cell layer) in whole-cell patch clamp experiments in rat hippocampal slices.

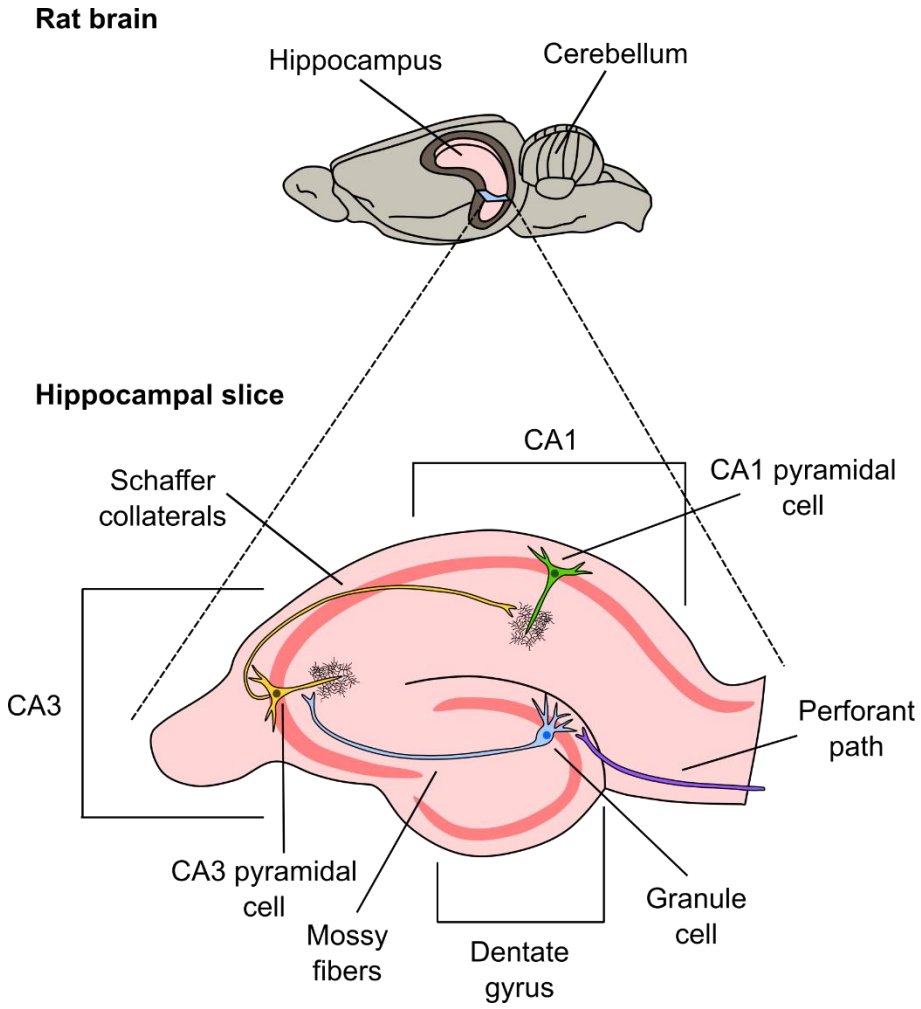


Figure 1-5. Rat hippocampal slice. Diagram showing a transversal hippocampal slice from rat brain and the main excitatory pathways. Adapted from (Purves et al. 2004).

The role of the hippocampus in learning and memory

The precise function of this particular structure of the human brain has been the object of study during the past few decades. Intensive research has finally led to two dominant theories on hippocampal function. The first one postulates a critical role of the hippocampus in the encoding and retrieval of memories related to facts and events, this is in long-term explicit or declarative memory (Squire 1992; Squire 2004). The second major theory is that the hippocampus plays a critical role in spatial memory, specifically hippocampal place cells that create cognitive maps used to navigate through space (O'Keefe & Nadel 1978; Burgess et al. 2002).

Declarative Memory Theory of hippocampal function. Evidence for the involvement of the hippocampus in declarative memory first came from the well-known case of the epileptic patient Henry Molaison (H.M) (Scoville 1954; Corkin 2002). H.M

underwent an unprecedented neurosurgical practice in which portions of the MTL, including the hippocampal formation, were removed in an attempt to relieve his otherwise intractable epileptic convulsions. Although the procedure considerably reduced the appearance of the seizures, it resulted in a severe and specific memory impairment. Whereas memory of early life events as well as working memory (short-term memory) and general cognitive abilities remained intact, H.M. was unable to acquire long-term memories for new facts or events after the surgery (anterograde amnesia). In other words, H.M. could not consolidate newly learned information (Scoviille & Milner 1957). This represented the first solid link between long-term memory and MTL structures, particularly the hippocampal formation. Data from cases of amnesic patients like H.M and lesion studies in animal models of amnesia (Zola-Morgan et al. 1994; Aggleton & Brown 1999; Murray & Mishkin 1998; Beason-Held et al. 1999; Zola et al. 2000) further supported a primary role of the hippocampal formation in memory. This is currently considered one of the main roles of this brain structure and it is the first proposition of the Declarative Memory Theory (Zola-Morgan et al. 1986; Squire & Zola-morgan 1991; Squire 1992; Squire 2004). The second proposition is that this involvement in memory is selective to declarative memory, including semantic and episodic, which in humans can be consciously recalled (Cohen & Eichenbaum 1993). Indeed, functional magnetic resonance imaging (fMRI) studies, which allow linking anatomical regions to specific functions, have shown that MTL components are required for the encoding (Small et al. 2001; Davachi & Wagner 2002; Reber et al. 2002) and retrieval (Gabrieli et al. 1997; Eldridge et al. 2000; C. E. Stark & Squire 2000; C. E. L. Stark & Squire 2000; Stark & Okado 2003) of memories of new facts (semantic memory) and events (episodic memory) (Cohen et al. 1999). The third proposition defines the hippocampal formation as a part of the MTL memory system (Squire & Zola-morgan 1991). Within this system, each component has different functions, but it is collectively involved in the formation and temporal storage of declarative memories (Squire & Zola-morgan 1991; Squire 1992). Finally, the four proposition states the time-limited role of the hippocampus in memory (Zola-Morgan & Squire 1990). Thus, memories are initially stored in the hippocampus but, as time passes after learning, memory traces are stored in the neocortex, where memory is consolidated, and the hippocampus is no longer involved in the recall of these memories (Zola-Morgan & Squire 1990; Squire & Alvarez 1995; O'Reilly & Norman 2002; Dudai & Morris 2000).

Cognitive Map Theory of hippocampal function. The key finding that triggered the postulation of this theory was the discovery of place cells in the hippocampus of freely-moving rats by J. O'Keefe and J. Dostrovsky in the 70's (O'Keefe & Dostrovsky 1971). Before this theory emerged, it had been demonstrated that hippocampal cells fire at different frequencies, a distinctive electrical activity known as theta frequency firing, during the characteristic exploratory behaviour displayed by a rat in a new environment (Vanderwolf 1969; Ranck 1973). O'Keefe and J. Dostrovsky performed electroencephalographic (EEG) recordings in hippocampal pyramidal cells and discovered that some of these neurons only exhibit this firing when the animal is in a specific location of the space (O'Keefe & Dostrovsky 1971). These location-reactive neurons received the name of place-cells and were described as typically silent until the animal entered a specific place of the environment where the cell began to fire, hence termed place fields (O'Keefe & Dostrovsky 1971). Now it is known that place cells are part of a "locale" neural system in which spatial information about external landmarks is stored in the form of a cognitive map (O'Keefe & Nadel 1978). These are then stored in the hippocampus and are used for spatial navigation (O'Keefe & Nadel 1978). Despite the formulation of this theory was made upon EEG recordings in animals, it has extended to humans. Apart from pure navigation purposes, cognitive maps in humans are also related to the storage and recall of linguistic and episodic memories, a function that has been attributed to the left hippocampus (Neyman & Manahan-Vaughan 2008; O'Keefe 2003; Burgess et al. 2002).

It is important to mention that the classical view of the hippocampus as a "memory structure" does not exclude its involvement in other brain functions and processes other than learning and navigation. The hippocampus has been implicated in other aspects of behaviour (Strange et al. 2014), including anxiety (Jimenez et al. 2018) and behavioural inhibition (Kimble 1968), as well as in sensorimotor function (Bast & Feldon 2003; Grion et al. 2016), novelty discrimination (Kaplan et al. 2014), and stress and the hypothalamic-pituitary axis responses (Sapolsky 1985; Kim & Diamond 2002).

The hippocampus during AD

AD is a neurodegenerative disorder in which the predominant symptom is memory loss, that aggravates throughout the course of the disease. Considering the critical role of the hippocampal formation in declarative memory, the fact that

neuropathological changes in AD firstly affect the entorhinal cortex and the hippocampus is not surprising (Braak & Braak 1991). Indeed, one of the consequences of impairment of hippocampal function is deficiency in episodic memory (Barker et al. 2017; Piolino et al. 2009; Viard et al. 2012). Accordingly, AD patients are unable to remember autobiographical events and recent affairs and to learn new information, both impairments related to deficits in encoding and storing of information.

The anatomical progression of AD pathophysiology was described according to the distribution of NFTs throughout the brain over the course of the disease by Braak and Braak in 1991 (Braak & Braak 1991). This gave rise to a hierarchical staging system named Braak staging that is today a reference in neuropathological studies of AD. The six stages defined by this system are as follows (Braak & Braak 1991):

- Stages I and II (transentorhinal stages). NFTs appear in the entorhinal cortex and the field CA1 of the hippocampus.
- Stages III and IV (limbic stages). NFTs accumulation increases in entorhinal cortex and CA1 and extends to other areas of the hippocampus, the subiculum and parasubiculum, and amygadala.
- Stages V and VI (isocortical stages). NFTs severely affect the entire hippocampal formation with neuronal loss in the CA region and reach the isocortical association areas.

Not only the hippocampus is greatly affected by NFTs deposition, but it also experiences severe changes in its size over the course of AD. Indeed, volumetric MRI measurements have shown that hippocampal atrophy correlates well with neuronal numbers in the hippocampus (Bobinski et al. 2000) and that AD is associated with a loss in hippocampal volume (Jack et al. 1998), a morphological change that can be observed at pre-symptomatic stages (Convit et al. 1997).

In summary, over the past years it has become clear that the hippocampal formation is one of the first structures targeted by AD pathology. However, why this region is so vulnerable to the pathology and how does this start are questions that remain to be answered.

1.2.6 Synaptic dysfunction in AD

Considering the essential role of synaptic plasticity for memory processes, it is without question that its disruption can lead to significant memory impairments, as those characterising the first signs of AD. Indeed, abnormalities of synaptic transmission have been characterised as part of the neuropathology. Morphometric analysis of temporal and frontal cortices of AD patients showed a major loss of synapses that was greater than that of neurons (Davies et al. 1987). Supporting this initial finding, a reduction in synaptic density in the dentate gyrus of AD patients (Scheff et al. 1996) as well as alterations in synaptic transmission and plasticity in the hippocampus of transgenic mice models of AD were found both *in vitro* (Larson et al. 1999) and *in vivo* (Giacchino et al. 2000). The relevance of these findings becomes obvious considering that these quantitative *post-mortem* measurements correlate more robustly with cognitive impairments before death than numbers of NFTs, amyloid plaques and other indicative parameters of neuronal dysfunction (Terry et al. 1991).

The discoveries described above led to the formulation of the “synaptic hypothesis of AD”. This postulates that synapses are an early target of AD, prior to neuronal loss and the onset of gross neurodegeneration, which accounts for the subsequent memory impairment, the most common initial symptom in AD patients (Selkoe 2002). Much of the support for this concept comes from studies examining the impact of neurotoxic species of Aβ on synaptic function, specifically in brain regions that are key for memory, such as the hippocampus. For instance, electrophysiological recordings in hippocampal slices from transgenic mice models of AD have shown smaller EPSPs as well as failure in maintaining LTP, which was correlated with deficits in memory and learning in behavioural tasks (Larson et al. 1999; Moechars et al. 1999; Chapman et al. 1999). In addition, it has also been found a decreased basal synaptic transmission in slices from these mice lines (Hsia et al. 1999). One prominent constraint of these studies is the variability in results due to differences in age and genetic background of mice lines and as well as in electrophysiological protocols used. Nonetheless, they do provide consistent evidence that AD transgenic mice display significant deficits in synaptic function that occur before plaque deposition. An additional difficulty is the identification of the Aβ species responsible for these synaptotoxic effects as transgenic mice are likely to accumulate different forms of Aβ in the brain, including monomers, soluble and insoluble oligomers, and fibrils (Selkoe 2002). However, it has been possible

to isolate different species of Aβ either from natural or synthetic sources, that can then be used to specifically differentiate their effects on synaptic function (Cleary et al. 2005; Cheng et al. 2007). For instance, it has been found that intracerebroventricular injection of Aβ oligomers, but not fibrils, in adult rats blocks hippocampal LTP (Walsh et al. 2002). In support of this, numerous studies have proven the concept that soluble Aβ oligomers contribute to a greater extent than fibrils and/or plaques to synaptic plasticity and memory impairments (Selkoe 2008; Shankar et al. 2008; Tomiyama et al. 2010) and correlate better with cognitive decline (Mucke et al. 2000; Näslund et al. 2000; McLean et al. 1999). These findings in animal models have led to the obvious therapeutic approach of reducing synaptotoxic Aβ forms in the brain as a successful attempt to rescue the impairments in synaptic function and cognition in animal models of AD (Dewachter et al. 2002; Dodart et al. 2002).

In summary, the synaptic hypothesis of AD has brought to light the important concept that prior to the development of gross AD neuropathology, changes to hippocampal synaptic function take place, more likely due to the presence of soluble Aβ forms than to amyloid plaques accumulation. Therefore, research efforts are increasingly focusing on understanding subtle synaptic alterations occurring at early stages of AD, which may hold the key for preventing early cognitive dysfunction.

Alterations in neurotransmitter systems can greatly impact on neuronal function and synaptic transmission. Indeed, disturbance of these systems has been associated with AD and may contribute to deficiencies in synaptic transmission observed in neurodegenerative processes, even before neuronal loss occurs (Kandimalla & Reddy 2017). Specifically, cholinergic and glutamatergic systems, which play a central role in cognition, appear to be selectively affected by AD pathology (Butterfield & Pocernich 2003; Francis 2003). Supporting this idea, current treatments for AD aim to restore the function of these neurotransmitters (Yiannopoulou & Papageorgiou 2013). Although these drugs do not stop the progression of the disease, they can ameliorate the symptoms of AD patients, clearly showing a significant involvement of glutamate and acetylcholine-mediated transmission in AD.

Cholinergic system in AD

Cholinergic transmission is mediated by the neurotransmitter acetylcholine (ACh) and it is involved in various aspects of cognitive function, including attention (Turchi & Sarter 1997; Chiba et al. 1995) and memory (Everitt & Robbins 1997). The latter is mediated by cholinergic neurons in the basal forebrain that project to the hippocampus, particularly neurons in the medial septum and the vertical diagonal band of Broca that form the septo-hippocampal pathway (Mesulam et al. 1983). These areas have been implicated in spatial and working memory, as ablation of these neurons resulted in impairments in spatial navigation tasks in rats (Beninger et al. 1986; Dunnet et al. 1987; Hagan et al. 1988; Berger-Sweeney et al. 1994). In AD, NFTs have been observed in the basal forebrain together with marked neuronal loss (Perry et al. 1977; Spillane et al. 1977; White et al. 1977; Whitehouse et al. 1982). The depletion of cholinergic axons is accompanied by a decrease in the activity of choline acetyltransferase (ChAT) (Davies & Maloney 1976; Wilcock et al. 1982), the enzyme that synthesises ACh, therefore resulting in reduced ACh uptake in AD. Since these cholinergic abnormalities are correlated with dementia severity (Perry et al. 1978; Bierer et al. 1995; Wilcock et al. 1982), these early observations led to the formulation of the "cholinergic hypothesis of AD". This hypothesis postulates that accumulation of NFTs in the basal forebrain and the subsequent loss of cholinergic projections into the hippocampus are responsible, at least in part, for the cognitive dysfunction observed in AD (Contestabile 2011). Although this has been supported by the positive effects of AChEIs inhibitors on cognitive deficits associated with AD (Doody et al. 2001; Courtney et al. 2004), the fact that they only function as a symptomatic treatment, and not even for all AD patients (Connelly et al. 2005; Lemstra et al. 2007), has brought significant controversy around the cholinergic hypothesis of AD.

Glutamatergic system in AD

AD pathology affects several areas where glutamatergic neurons are located, such as the layers III and V of the neocortex and the hippocampus (Kowall & Beal 1991; Francis 2003). As opposed to a reduced cholinergic transmission, the effects of AD pathology on the glutamatergic system generally lead to an increased concentration of glutamate around the synapses. The mechanisms proposed to explain this increase are numerous. For instance, a reduced presynaptic release of glutamate has been supported by the observation of decreased levels of

presynaptic vesicular glutamate transporters 1 and 2 (VGLUT1 and 2), responsible for maintaining the levels of glutamate in synaptic vesicles (Fremeau et al. 2001), in the brains of AD patients (Kashani et al. 2008; Kirvell et al. 2006). This may be due to preferential accumulation of Aβ peptides in neurons expressing these transporters (Sokolow et al. 2012). In addition, a mechanism consisting in reduced glutamate clearance could also take place, as levels of excitatory amino acid transporters 1 and 2 (EAAT1 and 2), responsible for glutamate clearance from the synaptic cleft (Andersen et al. 2006; Shashidharan et al. 1994), are also reduced in the cortex and hippocampus of AD patients (Jacob et al. 2007; Scott et al. 2011; Masliah et al. 1996; Li et al. 1997). Importantly, these changes may be an early sign in disease pathology (Masliah et al. 1996).

Taken together, these AD-related changes to the glutamatergic system lead to an accumulation of glutamate at the synapses (Revett et al. 2013). This has a well-established excitotoxic effect since built-up glutamate activates NMDARs, triggering a receptor-mediated increase in intracellular calcium concentration (Dong et al. 2009). Elevation in calcium levels can alter cell homeostasis and has various consequences that lead to cell death, such as the activation of calpain (Vosler et al. 2008) and the mitochondrial apoptotic pathway (Alberdi et al. 2010), which eventually result in neuronal loss associated with AD (Dong et al. 2009).

The coexistence of glutamate and acetylcholine receptors in pyramidal neurons in the neocortex led to the idea that effects mediated by these neurotransmitters may act in combination to cause disturbances in synaptic transmission (Francis 2005). This is further supported by the approval of a combination therapy consisting in an acetyl cholinesterase inhibitor and NMDAR blocker for the treatment of AD (Alzheimer's Association 2018). Although it is not clear how neurotransmitter systems interact, it has become evident that AD neuropathology very likely involves complex interactions at different levels of these systems that collectively contribute to synaptic dysfunction.

1.3 mGluRs and mAChRs in AD: a metabotropic problem

As outlined above, glutamatergic and cholinergic transmission are severely affected by the pathological effects of AD, which may partially explain the cognitive deficits of the disease. Specifically, growing evidence supports that dysregulation of metabotropic receptors from the glutamatergic and cholinergic families play an

important role in neurodegenerative mechanisms (Thathiah & De Strooper 2011). As a consequence, targeting these receptors may be of therapeutic interest.

Both mGluRs and mAChRs are synaptic GCPRs coupled to $G_{q/11}$ and the activation of PLC and phosphoinositide hydrolysis (Huang & Thathiah 2015). This shared signalling cascade is also a common pathway affected in disease. Indeed, previous studies have shown that $G_{q/11}$-associated signalling is compromised in AD brains (Shiozaki & Iseki 2004; Albasanz et al. 2005). This is likely to affect APP processing through the activation of PKC (Caporaso et al. 1992; Buxbaum et al. 1990) and mitogen-activated protein kinase (MAPK)-extracellular signal-regulated kinase (ERK) (Mills et al. 1997), both events regulated by $G_{q/11}$ activation by mAChRs or mGluRs (Fisher 2012; Sokol et al. 2011; Thathiah & De Strooper 2011). However, compelling evidence is now suggesting that there may be other pathways by which synaptic GPCRs can mediate pathological pathways.

Out of all muscarinic receptor subtypes, mAChR1 is the most abundantly expressed in the hippocampus (Levey et al. 1991; Wei et al. 1994; Flynn et al. 1995). In this region, mAChR1 is involved in short term memory and memory consolidation (Anagnostaras et al. 2003), a process known to be impaired in AD (Levey 1996). Supporting a role of mAChR1 in AD, a study shown that agonism of the receptor rescued impairments in hippocampal-dependent memory in an AD mouse model (Caccamo et al. 2006). Also, that these effects were due to reduction in amyloid-β and tau pathologies (Caccamo et al. 2006). Supporting this, another study showed an increase in amyloid-β production and plaque formation as a result of the inactivation of mAChR1 (Davis et al. 2010), in line with the concept of GPCR regulation of APP processing.

The involvement of mGluRs in AD has also been linked to the regulation of APP processing. Specifically, activation of the mGluR5 subtype has been shown to stimulate APP secretion (Sokol et al. 2011). In addition to this, other roles for mGluR5 in the pathophysiology of AD have emerged. A large body of evidence supports that aberrant activation of mGluR5 can mediate several Aβ-induced deficits in synaptic function (Wang 2004; Shankar et al. 2008; Hu et al. 2014; Rammes et al. 2011). Further showing the importance of mGluR5 in Aβ pathology, mGluR5 appears to be required for the Aβ-mediated impairments in memory and learning displayed in animal models of AD (Um et al. 2013; Hamilton et al. 2016; Hamilton et al. 2014). Some of these effects may be related to the regulation of

NMDAR-mediated excitotoxicity and degeneration by mGluR5 (Kingston et al. 1999; O'Leary et al. 2000; Movsesyan et al. 2001), as this is a known mechanism activated by Aβ (Molnár et al. 2004; Miguel-Hidalgo et al. 2002; Shankar et al. 2007; Malinow 2012; Snyder et al. 2005; Doble 1999). These studies have positioned mGluR5 as a central mediator of synapse weakening pathways (**Figure 1-6)**.

Altogether, the above evidence highlights the importance of synaptic GPCRs, mAChRs and mGluRs, in the pathology of AD. Since members of both families are coupled to the same G-protein mediated signalling pathway, the possibility of a functional interaction between these receptors has emerged. Given that both receptors are separately implicated in AD pathology, their potential interplay could also play a role in how receptor function is altered in synaptotoxic environments.

The concept of cross-talk interaction between GPCR-mediated pathways is not new, however (Hur & Kim 2002). This concept is based on the idea that G-protein subunits can exert competitive stearic hindrance when binding to their receptors (Hippe et al. 2013). For this to occur, simultaneous activation of both GPCRs coupled to the same specific G-proteins is presumed, as they are present in a limited amount in the intracellular space (Thompson et al. 2007). Since mGluR5 is aberrantly activated under AD-like conditions and deficiency in cholinergic transmission is a hallmark of AD pathology, the potential contribution of mGluR5-mediated inhibition of mAChRs remains possible. Supporting this functional inhibition hypothesis, aberrant expression of mGluR5 leads to the inhibition of mAChR-mediated LTD in the perirhinal cortex (Jo et al. 2006). Furthermore, preliminary data from our lab have shown that in the same brain region, the Aβ-mediated inhibition of mAChR-dependent LTD occurs in a mGluR5-dependent manner (unpublished results). In line with this, our data also shows that dexamethasone-mediated inhibition of mAChRs-dependent LTD is also dependent on group I-mGluRs activation (unpublished results).

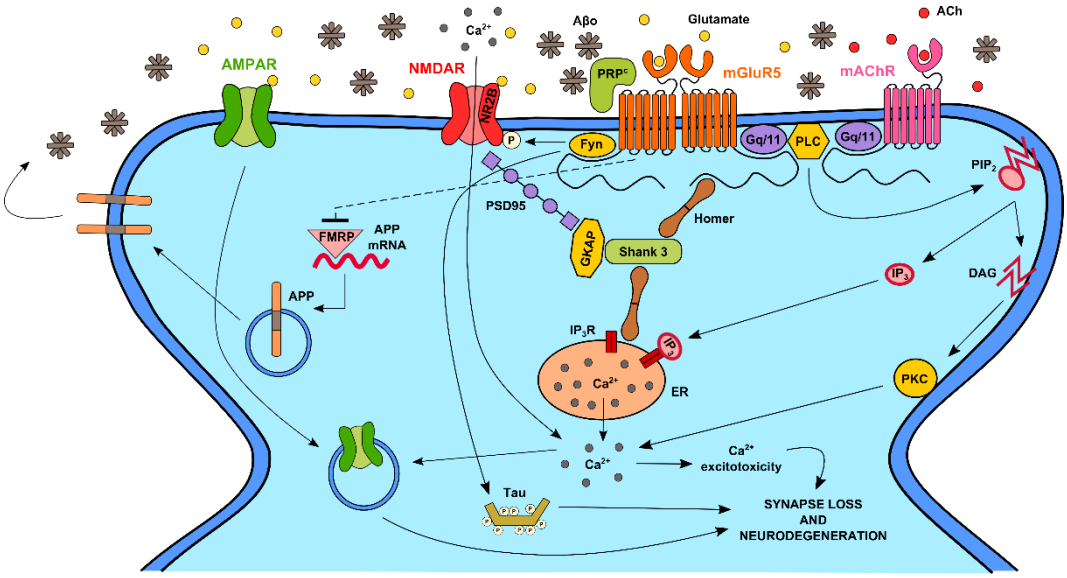


Figure 1-6. Model of synaptic dysfunction in AD. Under pathological conditions, overactivation of mGluR5 leads to increased intracellular calcium levels and the generation of calcium excitotoxicity. This triggers multiple pathological mechanisms that result in synapse loss and subsequent neurodegeneration. This effect is enlarged by Aβ-induced activation of mGluR5-PrP^C signalling complex which results in Fyn-dependent phosphorylation of NMDARs as well as phosphorylation of tau. Both events trigger pathological signalling cascades including removal of AMPAR from the synaptic membrane and disassembly of hyperphosphorylated tau from microtubules, that together contribute to synapse weakening pathways. In addition, activation of mGluR5 results in an increased production of toxic Aβ species via blockade of the repressive effect of FMRP on the translation of APP mRNA. Finally, competition between mGluR5 and mAChR for shared signalling molecules is a potential mechanism by which cholinergic function is impaired in AD pathology. Dashed arrow indicates unknown mechanism. ER: Endoplasmic reticulum, APP: Amyloid-precursor protein, AMPAR: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor, NMDAR: N-methyl-D-aspartate receptor, mGluR5: metabotropic glutamate receptor 5, mAChR: muscarinic acetylcholine receptor, ACh: acetylcholine, IP_3: inositol 1,4,5-triphosphate, PKC: protein kinase C, DAG: diacylglycerol, PIP_2: phosphatidylinositol 4,5-bisphosphate, PrP^C: cellular prion protein, GKAP: guanylate kinase-associated protein, FMRP: Fragile X mental retardation protein.

In summary, compelling evidence positions synaptic GPCRs dysregulation as a key to understand the pathophysiology of AD. Specifically, mGluR5 and mAChR1 have become very attractive targets in AD therapeutics. However, a better understanding of the mechanisms mediated by these receptors in disease is required in order to develop efficient treatments for AD.

1.4 Hypothesis and aims

Based on **(1)** evidence showing a role of synaptic GPCRs, mGluR5 and mAChR1, in the synaptic impairments that characterise AD pathophysiology (Wang 2004; Hu et al. 2014; Caccamo et al. 2006) and **(2)** preliminary data from this lab showing

an impairment of mAChRs function mediated by mGluR5 (unpublished results), the general hypothesis of this thesis is that:

Aberrant activation of mGluR5 has detrimental effects on synaptic function and can lead to the impairment of mAChR receptor function, and these effects are enhanced in AD pathology.

Specific hypotheses are stated within each aim below.

Aim 1: Characterise the role of mGluR5 in synaptic plasticity in non-pathogenic conditions. It was hypothesised that agonist activation of mGluR5 causes the following effects: **(1)** depression of baseline recordings and blockade of LTP induction in acute hippocampal slices, **(2)** reduction of mAChR-mediated calcium increase elicited by application of the mAChR agonist carbachol (CCh) in primary hippocampal cultured neurons and **(3)** activation of GSK3β in acute hippocampal slices.

Aim 2: Characterise the expression of synaptic proteins over AD progression in *post-mortem* human brain samples. It was hypothesised that **(1)** mGluR5 protein expression was increased in brains from patients with severe AD and that **(2)** mAChR1 protein expression was decreased in brains from patients with severe AD compared to control brains.

Aim 3: Characterise the effect of AD pathology in the form of hyperphosphorylated human tau on synaptic GPCRs function. Based on results from human brain work, it was hypothesised that expression of P-hTau in cultured hippocampal slices **(1)** reduces the mAChR-mediated change in holding current elicited by CCh and **(2)** does not reduce the mGluR-mediated change in holding current elicited by the mGluR agonist, DHPG.

1.5 Summary

The general aim of this thesis is to investigate the roles of mGluRs and mAChRs in AD pathology. **Chapter 3** is focused on understanding whether mGluR5 activation is sufficient to cause effects related to AD pathology, such as the impairment of LTP and mAChR function and the activation of GSK3β. **Chapter 4** looks at the state of receptors in AD brains, to evaluate whether the findings from

Chapter 3 may be relevant in disease. **Chapter 5** then explores if tau hyperphosphorylation, a hallmark of AD brains, can affect synaptic GPCR function. Finally, **Chapter 6** provides an overall discussion about how these results can be explained within the context of AD pathophysiology.

Chapter 2 Materials and methods

2.9 Co-immunoprecipitation (Co-IP) assay with human tissue

2.9.1 Tissue homogenisation

2.9.2 Wash of protein-G agarose beads

2.9.3 Pre-clearing of lysates

2.9.4 Pull-down antibody incubation

2.9.5 Protein-G agarose beads incubation

2.9.6 Washing steps

2.9.7 Protein elution and gel loading

2.10 Biolistic transfection of organotypic cultured hippocampal slices

2.10.1 DNA constructs for biolistic transfection

2.10.2 Precipitation of DNA into gold microcarriers

2.10.3 Loading the DNA/microcarrier suspension into tefzel tubing: Tubing Prep Station

2.10.4 Preparation of cartridges

2.10.5 Biolistic transfection

2.11 *In vitro* electrophysiological recordings in rat slices

2.11.1 Equipment set-up

2.11.2 Recording and stimulating electrodes

2.11.3 Extracellular field recordings

2.11.4 Intracellular whole-cell patch clamp recordings

2.11.5 Electrophysiological data acquisition, analysis and statistics

2.12 Pharmacological agents and treatments

2.1 Animals

Male Wistar rats at postnatal day (P) 24-30 (Charles River, UK) were used for the preparation of acute hippocampal slices. Animals were housed in groups of 4-5 per cage with water and food *ad libitum*. Cages were maintained at 21 ± 2°C and relative humidity of 55 ± 10%. Animals were exposed to a 14 h light/10 h dark cycle with the light phase starting at 5:00 h and finishing at 19.00h. P7 male Wistar rats were used for the preparation of organotypic hippocampal slice cultures and P0 male Wistar rats were used for the preparation of primary neuronal cultures. Adult female Wistar rats were used for a maximum of 6 litters and they were time mated by checking for plugs. Females were kept alone until they had their litter. All efforts were made to minimize numbers of animals used as well as animal suffering. All procedures involving animals were performed in accordance with the UK Animals Scientific Procedures Act of 1986.

2.2 *In vitro* preparations

2.2.1 Acute hippocampal slices preparation from rat brain

Acute hippocampal slices were prepared from P24-30 male Wistar rats. Animals were sacrificed between 10:00 a.m. and 11:00 a.m. by cervical dislocation and then decapitated with scissors. The skull was cut following the longitudinal fissure and each part was separated. The brain was rapidly removed and placed in ice-cold artificial cerebrospinal fluid (aCSF) bubbled with 95% O_2/5% CO_2, which comprised 124 mM NaCl, 3 mM KCl, 26 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 2 mM $CaCl_2$, 1 mM $MgSO_4$ and 10 mM D-glucose. The two hemispheres were then separated by performing a midsagittal cut of the brain and one hemisphere was placed back into the ice-cold aCSF to be dissected subsequently. The hippocampus was micro-dissected from the whole hemisphere. First, the hemisphere was placed with the dorsal side facing down so the ventral side could be observed. Gentle pressure was applied to the front part of the hemisphere to hold it in place while the cerebellum was folded back with forceps. Next, a spatula was placed in the lateral ventricle and swept underneath each end of the hippocampus to separate it from adjacent cortex tissue. With the spatula underneath the hippocampus, a 180° turn was performed to lift it out. Finally, the hippocampus was cut away from remaining cortex tissue by applying pressure with the spatula while moving it along the dorsal edge of the hippocampus. The hippocampus was then placed on a filter paper and transversely sliced using a McIlwain tissue chopper (The Mickle Laboratory

Engineering Co. Ltd., Gomshall, UK) to obtain 400 µM thickness hippocampal slices. Slices were re-submerged in ice-cold aCSF bubbled with 95% O_2/5% CO_2 and separated. Finally, slices were placed in a submerged chamber containing aCSF at room temperature (RT: ~21°C) and allowed to recover for at least 1 hour.

2.2.2 Organotypic cultured hippocampal slices preparation from rat brain

Organotypic hippocampal slice cultures were prepared from P7 Wistar rats. Dissection areas and sterilised surgical tools were sprayed with 70% ethanol to ensure sterile conditions before starting the procedure. Rats were sacrificed by dislocation of the neck and then decapitated with scissors. The skull was cut following the longitudinal fissure and each part was separated. The brain was rapidly removed and placed in ice-cold cutting solution (previously filtered in sterile conditions) that contained: 238 mM sucrose, 2.5 mM KCl, 26 mM $NaHCO_3$, 1 mM NaH_2PO_4, 11 mM D-glucose, 5 mM $MgCl_2$ and 1 mM $CaCl_2$. At this point, the procedure was performed in sterile conditions in a laminar-flow hood. The two hemispheres were separated by performing a midsagittal cut of the brain and one hemisphere was placed back into the ice-cold cutting solution to be dissected subsequently. The hippocampus was micro-dissected from the whole hemisphere in accordance to the method described for acute hippocampal slices. The hippocampus was transversely sliced using a McIlwain tissue chopper (The Mickle Laboratory Engineering Co. Ltd., Gomshall, UK) to obtain 350 µM thickness hippocampal slices. To remove cutting solution, slices were washed four times in cultured medium (previously filtered in sterile conditions) comprising: 78.8% minimum essential medium, 20% heat-inactivated horse serum, 30 mM HEPES, 26 mM D-glucose, 5.8 mM $NaHCO_3$, 2 mM $CaCl_2$, 2 mM $MgSO_4$, 70 µM ascorbic acid, 0.1% 1 mg/ml insulin, pH 7.3 and 320-330 mOsm. Slices were then placed on semi-permeable membrane inserts (Millipore Corporation, Bedford, MA, USA) in a six-well plate and the excess of culture medium on the membrane insert was removed with a pipette. On initial plating, each well contained 950 µL of culture medium pre-warmed at 37°C. Medium was changed every 2 days without antibiotics with a pipette, avoiding direct application of medium on the slices. First change of medium was done with 900 µL of fresh medium and subsequent changes with 850 µL. Six-well plates were kept in the incubator at 37°C with 20% O_2, 5% CO_2.

2.2.3 Primary neuronal culture preparation from rat brain

Primary neuronal cultures were prepared as described in (Brewer & Torricelli 2007) with some variations. Hippocampal and cortical neurons were dissected from P0-1 Wistar rats (Charles River, UK) using sterile surgical tools. Rats were sacrificed by decapitation and the brain was removed and transferred to Hibernate-A medium (A1247501, Thermo Fisher) supplemented with B-27 Supplement serum-free (17504044, Thermo Fisher) and GlutaMAX (35050038, Thermo Fisher), hereafter termed HABG medium. The cerebellum and brainstem were removed, the cortex and hippocampus were isolated from the midbrain and meninges were removed. At this point, the procedure was performed in sterile conditions in a laminar-flow hood. Tissue was pulled apart into pieces of approximately 2 mm^3 and digested with Trypsin-EDTA (25200056, Thermo Fisher) by addition to Hibernate-A/GlutaMAX medium for 15 min at 37°C. Trypsin was inactivated by addition of HABG and the tissue was further dissociated by pipetting tissue into and out of a Pasteur pipette with a fire-polished tip. The cell suspension was added to a prepared OptiPrep Density Gradient Medium (D1556, Sigma-Aldrich) in HABG and centrifuged at 160 g for 12 minutes. OptiPrep was used for separation of cells according to their density. The resulting upper two fractions contained debris and oligodendrocytes and were discarded. The third fraction was enriched for neurons which were quantified using Trypan-blue exclusion in a haemocytometer. Neurons were transferred to Neurobasal-A medium (10888022, Thermo Fisher) supplemented with B-27, GlutaMAX and gentamycin (15710049, Thermo Fisher). Neurons were plated into 10 mm diameter glass coverslips coated with poly-D-lysine (P7280, Sigma-Aldrich) at a density of 3 x 104 cells/cm^2. Coverslips were placed in 12-well plates (665180, Greiner Bio-One), each well containing 1 mL of supplemented Neurobasal-A medium. This medium was changed at 21 days by replacing half of it with 500 µL of fresh medium. Neurons were kept in the incubator at 20% O_2, 5% CO_2 at 37°C. Thank you to Tom Steward for performing this procedure.

2.3 Human *post-mortem* brain samples

Two sets of human samples were obtained from the South West Dementia Brain Bank (Southmead Hospital, Bristol Medical School, Faculty of Health Sciences, University of Bristol). Diagnosis of patients was performed by following a combined clinical and neuropathological assessment according to (Mirra et al. 1991) and the

National Institute of Aging-Alzheimer's Association (NIA-AA) (Hyman et al. 2012). These criteria incorporate the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) scale, which is a score system based on a series of standardised and validated measurements for the assessment of AD (Fillenbaum et al. 2008). These include clinical, neuropsychology, neuropathology, neuroimaging, behavioural, family history, autopsy and educational assessments. The semi-quantitative evaluation of the frequency of age-related plaque score (**Table 2**) together with the clinical history, results in a score used as an indication of the certainty of the diagnosis of AD, i.e. definite, probable AD, possible AD, no evidence of AD (Hyman et al. 2012) (**Table 3**). The progression of AD was measured according to the Braak staging method, which is a topographical staging of the NFTs deposition in the brain. Since the spatiotemporal pattern of progression of NFTs is stereotypical and predictable and correlates with the severity of cognitive decline (Braak & Braak 1991), this system is used for the pathological diagnosis of AD. Broadly, the transentorhinal and hippocampal regions are first affected (stages I-II), then the limbic areas (stages III-IV) and finally isocortical areas (V-VI) (Serrano-Pozo et al. 2011).

Table 2. Age-related plaque score.

	Frequency of plaques			
Age of patient at death (years)	**None**	**Sparse**	**Moderate**	**Frequent**
<50	0	C	C	C
50-75	0	B	C	C
>75	0	A	B	C

0 = No histologic evidence of Alzheimer's disease.
A = Histologic findings are uncertain evidence of Alzheimer's disease.
B = Histologic findings suggest the diagnosis of Alzheimer's disease.
C = Histologic findings indicate the diagnosis of Alzheimer's disease.
Thank you to South West Dementia Brain Bank for providing this information.

The first set of samples, hereafter referred as "AD samples", contained temporal lobe cortex samples from subjects with definite AD (Braak stages V-VI, N = 10) and non-demented aged matched control subjects (Braak stages 0-III, N = 10). The second set, hereafter referred as "P-AD samples", contained temporal lobe cortex samples from subjects with probable/possible AD (Braak stages III-IV, N = 11) and non-demented aged matched control subjects (Braak stages 0-III, N = 11). All human tissue samples were handled according to the regulations of the Human Tissue Authority (HTA) of the Department of Health of the UK Government.

Available clinical and neuropathological data regarding human tissue brain samples is shown in **Table 4** and **Table 5**.

Table 3. CERAD diagnostic groups.

CERAD diagnostic group	Description
Normal (with respect to dementia)	No histologic evidence of AD (0 score) *and* no clinical history of dementia, *and* absence of other neuropathologic lesions likely to cause dementia *or* An A age-related plaque score *and* no clinical history of dementia
Definite AD	C age-related plaque score *and* clinical history of dementia, *and* presence or absence of other neuropathological lesions likely to cause dementia
Probable AD	B age-related plaque score, *and* clinical history of dementia, *and* presence or absence of other neuropathologic lesions likely to cause dementia
Possible AD	A age-related plaque score, *and* clinical history of dementia, *and* presence or absence of other neuropathologic lesions likely to cause dementia *or* B or C age-related plaque score *and* absence of clinical manifestations of dementia

Thank you to South West Dementia Brain Bank for providing this information.

Table 4. Clinical and neuropathological data of AD human samples.

Group	MRC ID[1]	Sample	Age	Gender	PM delay[2]	CERAD scale	Braak stage
C	BBN_9292	C1	73	M	35	n/a	III
	BBN_9329	C2	80	M	45.75	n/a	0
	BBN_4205	C3	87	M	24	n/a	II
	BBN_4229	C4	87	F	47	n/a	III
	BBN_9399	C5	73	F	50	n/a	II
	BBN_9407	C6	90	F	41	n/a	II
	BBN_9413	C7	82	M	67	n/a	II
	BBN_22625	C8	70	F	33.25	n/a	II
	BBN_24312	C9	69	M	31.25	n/a	II
	BBN_26009	C10	87	F	54.75	n/a	II
AD	BBN_9371	AD1	73	F	50.5	Definite AD	V
	BBN_9377	AD2	75	M	39.5	Definite AD	V
	BBN_9401	AD3	86	F	45.25	Definite AD	VI
	BBN_9405	AD4	88	M	28	Definite AD	VI
	BBN_9435	AD5	74	M	45.5	Definite AD	V
	BBN_22622	AD6	81	F	58.25	Definite AD	V
	BBN_24563	AD7	67	M	36	Definite AD	VI
	BBN_26011	AD8	80	M	68.5	Definite AD	IV
	BBN006.26796	AD9	66	F	37.25	Definite AD	VI
	BBN006.28766	AD10	87	F	40.5	Definite AD	V

Table 5. Clinical and neuropathological data of P-AD human samples.

Group	MRC ID[1]	Sample	Age	Gender	PM delay[2]	CERAD scale	Braak stage
C	BBN_24561	C1	90	F	67.25	No AD	I
	BBN_8712	C2	81	F	103	No AD	II
	BBN_9353	C3	94	M	40	No AD	II
	BBN_9407	C4	90	F	41	No AD	II
	BBN_9408	C5	87	M	42	No AD	II
	BBN_9432	C6	74	F	27.5	No AD	II
	BBN_10251	C7	79	F	48	No AD	II
	BBN_26009	C8	87	F	54.75	No AD	II
	BBN_8949	C9	79	M	24	No AD	0
	BBN_19626	C10	81	M	35.75	No AD	III
	BBN_9311	C11	93	M	37.75	No AD	III
P-AD	BBN_9331	P-AD1	97	M	34	Possible AD	IV
	BBN_9343	P-AD2	80	M	24	Probable AD	IV
	BBN_4215	P-AD3	80	F	26	Probable AD	IV
	BBN_4216	P-AD4	84	F	48	Probable AD	IV
	BBN_9394	P-AD5	81	M	32	Probable AD	IV
	BBN_9433	P-AD6	88	M	36	Probable AD	IV
	BBN_9257	P-AD7	82	F	110	Probable AD	IV
	BBN_4220	P-AD8	86	F	13.5	Possible AD	III
	BBN_9397	P-AD9	97	F	36	Possible AD	III
	BBN_22624	P-AD10	93	M	44	Probable AD	III
	BBN_9217	P-AD11	93	F	53	Probable AD	III

[1] **Medical Research Council ID,** [2] ***Post-mortem*** **(PM)** delay refers to the time interval in hours between the time of the death and the time when the brain is frozen**.** C: Non-demented aged-matched control subject, AD: Alzheimer's disease patient, P-AD: Possible/probable AD patient, M: Male, F: Female, n/a: not available.

For technical purposes and to facilitate the manipulation of human samples when loading polyacrylamide gels (see **Section 2.8.3**), the Medical Research Council ID of each sample was randomly assigned a sample name consisting of a letter ("C" for non-demented aged-matched control subject, "AD" for Alzheimer's disease patient and "P-AD" for possible/probable AD patient) and a number (from 1 to 10 within the AD cohort and from 1 to 11 within the P-AD cohort).

2.4 Total RNA extraction from human tissue

Total RNA was extracted from *post-mortem* human tissue samples by acid phenol-guanidinium-thiocyanate chloroform extraction (Chomczynski & Sacchi 1987) as described in (Anon 2006) with some variations. Approximately, 50 mg of tissue were homogenised in 1 mL of solution D (4 M guanidinium thiocyanate, 25 mM

sodium citrate $2H_2O$, 0.5% (wt/vol) sodium lauryl sarcosinate, 0.1 M 2-mercaptoethanol) using a polypropylene pellet pestle (Z359947, Sigma) and a pellet pestle motor (Kimble® Kontes, Z359971, Sigma) for 30 s. Then, 100 µL of 2 M sodium acetate, 1 mL of phenol and 200 µL of 49:1 (wt/vol) chloroform/isoamyl alcohol were added per mL of solution D, mixing the contents after addition of each reagent by inversion of the tubes. Samples were incubated 10 min on ice and centrifuged at 10,000 g for 20 min at 4°C. The aqueous phase was transferred to a fresh tube and an equal volume of isopropanol (~900 µL) was added to the extracted RNA. Tubes were then thoroughly mixed and RNA was allowed to precipitate for 1 h at -20°C. After this period, RNA was collected by centrifugation at 10,000 g for 30 min at 4°C. Isopropanol was decanted and the RNA pellet was dissolved in 0.3 mL of solution D for every mL of this solution added in the first step. An equal volume of isopropanol was added and RNA was precipitated for 1 h at -20°C. Precipitated RNA was collected by centrifugation at 10,000 g for 10 min at 4°C. The resulting pellet was washed twice with 75% ethanol, carefully removing any remaining ethanol in the second wash. Samples were allowed to air dry for 10 min and then dissolved in 50 µL of 0.1% diethylpyrocarbonate (DEPC)-treated water. Finally, they were incubated at 50°C for 10 min to fully dissolve the pellets. Absorbance of samples was measured at 260 and 280 nm by using a NanoPhotometer (IMPLEN, München, Germany) and RNA concentration was determined by the Beer-Lambert law. Purity of samples was assessed by the A260/280 ratio. RNA samples were aliquoted and stored at -80°C for further use.

2.5 First-strand cDNA synthesis from human RNA samples

For cDNA synthesis, 1 µg of extracted RNA was reverse transcribed using SuperScript™ III Reverse Transcriptase kit (18080-44, Thermo Fisher Scientific) following the manufacturer's instructions. First, RNA samples were diluted in DEPC-treated water to obtain 1 µg of RNA in a total volume of 11 µL in a nuclease-free reaction tube. A mixture containing 1 µL of 50 µM oligo(dT)20 (18418-020, Thermo Fisher Scientific) and 1 µL of 10 mM dNTP mix (18427-013, Thermo Fisher Scientific) was prepared (quantities indicated per RNA sample). Then, 2 µL of this mixture was added to the reaction tube. Reaction tubes were heated at 65°C for 5 min and immediately incubated on ice for at least 1 min. Contents were then collected by brief centrifugation. A mixture of 4 µL of 5X First-Strand Buffer, 1 µL of 0.1 M DTT, 1 µL of RNaseOUT™ Recombinant RNase Inhibitor (10777-019, Thermo Fisher Scientific) and 1 µL SuperScript™ III RT (quantities indicated per

RNA sample) was prepared and gently mixed by pipetting up and down. Then, 7 µL of this mixture were added to each reaction tube and mixed by gentle pipetting. A minus reverse transcription (-RT) control tube in which the 1 µL SuperScript™ III RT was replaced by 1 µL of DEPC-treated water was prepared with one randomly selected sample per each cDNA synthesis procedure. This control is to test for contaminating DNA during the RNA extraction procedure. If there is amplification of product detected in the qPCR step, it is most likely derived from contaminating DNA, as the -RT control tube should not be amplified. In total, each cDNA reaction volume was 20 µL. In the thermal cycler (T100, Bio-Rad, California, United States), reaction tubes were incubated at 50°C for 1 h and then the reaction was inactivated at 70°C for 15 min. Resulting cDNA was stored at -20°C until required.

2.6 Quantitative polymerase chain reaction (qPCR) with human cDNA

For qPCR reactions, a master mix was prepared including (per cDNA sample): 5 µL of Fast SYBR Green Master Mix (4385612, Thermo Fisher Scienctific), 1.5 µL of 2 µM forward primer, 1.5 µL of 2 µM reverse primer and 1 µL (see **Table 6**) of nuclease-free water. After a light vortex, 9 µl of this mix was added to each well of a MicroAmp™ Fast Optical 96-Well Reaction Plate (4346906, Thermo Fisher Scientific). Then, 1 µL of cDNA was added to each well and mixed by pipetting up and down three times. Plates were always placed on a MicroAmp™ 96-Well Support Base (4379590, Thermo Fisher Scientific) and never in contact with the bench surface to prevent dirt particles from adhering to the bottom of the wells and obstructing the fluorescence reading. To generate a standard curve, a serial dilution (Neat - 1:10 - 1:100 - 1:1000 - 1:10,000 - 1:100,000) of cDNA synthesised from human brain total RNA (AM7962, Life Technologies) was performed. One standard curve was generated per plate. Each plate included a no-RT control sample (see cDNA synthesis section above) and a no-template control (NTC) in which the 1 µL of cDNA sample was replaced by 1 µL of DEPC-treated water. The function of the NTC was to test for primer dimer formation and for DNA contamination in the reagents or the plate. The total volume of the qPCR reaction was 10 µL (each well) and reactions were run in triplicates. Once loaded, plates were covered with a MicroAmp™ Optical Adhesive Film (4360954, Thermo Fisher Scientific) and spun in a plate spinner for a few seconds to ensure contents were at the bottom of the well. qPCR reactions were performed in a StepOnePlus™

Real-time PCR System (Applied Biosystems, California, USA). The plate was heated at 95°C for 20 s (holding stage) followed by 40 cycles of 3 s at 95°C - 30 s at 60°C (cycling stage). After the last cycle was completed, melt curve stage was performed for 15 s at 95°C - 1 min at 60°C - 15 s at 95 °C. Data was visualised in real time using the StepOne v2.3 software (Applied Biosystems-Life Technologies©).

Table 6. Primers used in this study.

Gene (Protein)	Accession Nº	Primers (5' → 3')	GC (%)	T_m (°C)
ACTB (β-Actin)	NM_001101	For: AGAGCTACGAGCTGCCTGAC Rev: AGCACTGTGTTGGCGTACAG	61 55	60
GUSB (β-Glucuronidase)	NC_000007.14	For: CTAACTATGCAGCAGACAAGG Rev: GATACCAAGAGTAGTAGCTGTTC	44 43	57
RPL13 (RPL13)	NM_000977	For: CCTGGAGGAGAAGAGGAAAGAGA Rev: TGAGGACCTCTGTGTATTTGTCAA	52 40	60
GRM5 (mGluR5)	D28539	For: CCAGCACAAGTCGGAAATAGAG Rev: TGCTCATTGTTGCTCTCCCAC	50 52	60
GRIA2 (GluA2)	NM_000826.3	For: GAAGTAAGGAAAAGACCAGTGCC Rev: CCACCTTCATTCGTTTCGCC	47.83 55	60
DLG4 (PSD95)	NM_001128827	For: CCCCCAACATGGACTGTCTC Rev: CGGTCCCGTTCACATATCCT	60 55	60

Thank you to Dr. Felicity Stubbs (Lightman's Lab) for providing GUSB primers. For: Forward primer, Rev: reverse primer.

2.6.1 qPCR data analysis and statistics

Relative product quantities were calculated using the relative standard curve method (Anon 2013; Larionov et al. 2005). A standard curve was generated with relative quantities and CT values from diluted human brain total cDNA samples. For the experimental samples, relative quantity values were interpolated from the standard curve and normalised to the endogenous control gene, ribosomal protein 13 (RPL13). Statistically significant differences ($p < 0.05$) between patient groups were tested by using Mann-Whitney rank sum test, given the non-normal distribution of the data.

2.7 Calcium imaging in primary cultured hippocampal neurons

The following procedure was performed in sterile conditions in a laminar-flow hood. 12-well plates were taken out of the incubator and placed in the laminar-flow hood and Neurobasal-A medium was removed with a pipette. Coverslips were washed four times with 500 µL of HBS buffer (119 mM NaCl, 5 mM KCl, 25 mM HEPES,

33 mM glucose, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 1 µM glycine, 100 µM picrotoxin, pH ~7.4) supplemented with BSA (1 mg/mL). Fluo-4AM (F14201, Life Technologies), a cell-permeant calcium fluorescent dye (λEx/λEm of calcium–bound form: 494/506 nm), was prepared at 5 µM in HBS/BSA in the dark. Neurons were loaded with 250 µL of Fluo-4AM-containing HBS/BSA solution at 20% O_2, 5% CO_2, 37°C in the dark for 1 h. Coverslips were subsequently washed 3 times with 500 µL of HBS and placed in the perfusion chamber for imaging where they were constantly perfused with HBS buffer (flow rate ~2 mL/min) throughout the experiment. Neurons were viewed through a 20X lens in an inverted microscope (Leica DM IRBE, Wetzlar, Germany) equipped with a stage (OptiScan II, Prior Scientific, Cambridge, UK) and a digital camera (ORCA 100 C4742-95, Hamamatsu Photonics, Hamamatsu, Japan) with a charge-coupled device (CCD) for image acquisition. Fields with 4 or more neurons were selected on bright-field mode and then Fluo-4AM fluorescence was detected on epifluorescence mode using a mercury lamp as a light source. Images were taken every 20 seconds and visualised on a computer screen using the imaging software SimplePCI (Hamamatsu Photonics, K.K, ©2017). An overview of the calcium imaging experimental procedure is depicted in **Figure 2-1.**

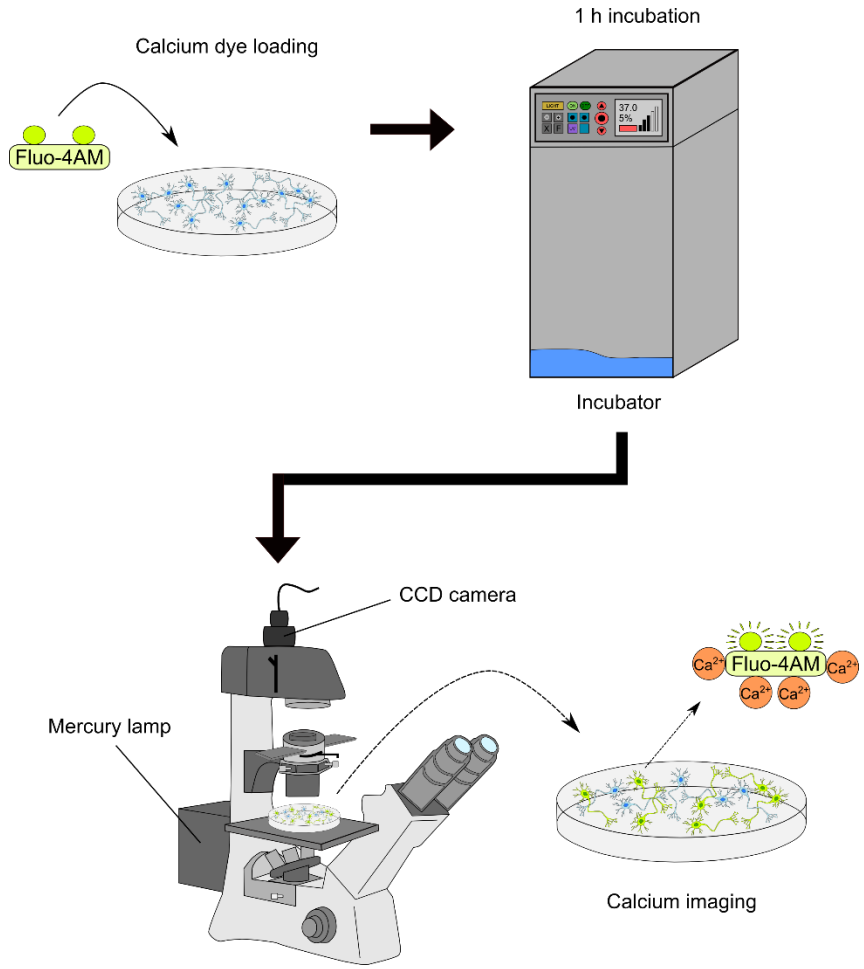


Figure 2-1. Schematic diagram of calcium imaging experiments. Primary cultured cells were loaded with the calcium dye Fluo4-AM for 1h at 37°C in the dark. After a series of washes, coverslips were placed on an inverted microscope equipped with a mercury lamp for visualisation of fluorescent signals and a CCD camera for image acquisition.

2.7.1 Calcium imaging data analysis and statistics

Image analysis was performed using Icy (Institut Pasteur, ©2011). Regions of interest (ROIs) were selected according to soma morphology and fluorescence intensity values were obtained for each ROI and for a background region (a region with no neurons) using ROI Intensity Evolution tool. For each neuron, background intensity was subtracted from ROI intensity at each time point. Data were normalised to the average of the pre-conditioning baseline (5 min) and expressed as a percentage of this baseline. For each experiment (i.e. coverslip), the average of peak intensity values from neurons within a coverslip was obtained and data across experiments was pooled and plotted in the graphs. Statistically significant differences ($p < 0.05$) between treatments were tested by using Kruskal-Wallis one-way ANOVA on Ranks or Mann-Whitney rank sum test, given the non-normal distribution of the data.

2.8 Protein extraction and western blot of rat slices and human tissue

2.8.1 Protein extraction from acute hippocampal slices

This procedure was performed at 4°C by keeping samples and lysis buffer on ice at all times. Acute hippocampal slices were collected in tubes with aCSF from the incubation chamber. Lysis buffer comprised RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% Triton, 0.5% sodium deoxycholate, 0.1% SDS, 1mM EDTA) with the addition of protease inhibitor cocktail at 1:10 (05892791001, Roche), phosphatase inhibitor cocktail at 1:100 (P5726, Sigma) and the serine/cysteine protease inhibitor phenylmethanesulfonyl fluoride (PMSF) at 1:100 (P7626, Sigma). First, aCSF was removed as much as possible without damaging the slice, then lysis buffer (100 µL/slice) was added and the tissue was homogenised using a polypropylene pellet pestle (Z359947, Sigma) and a pellet pestle motor (Kimble® Kontes, Z359971, Sigma) for 30 s. Samples were then centrifuged at 15,000 g for 10 min at 4°C. Pellets were discarded and supernatants were collected in a fresh tube. The protein concentration of these samples was determined using a bicinchoninic acid (BCA) assay (Pierce™ BCA Protein Assay Kit, 23225, Thermo Fisher Scientific). Absorbance of the samples was measured at 565 nm with iMark Microplate Absorbance Reader (Bio-Rad, California, USA). Microplate Manager v6 software (Bio-Rad, California, USA) was used to acquire absorbance measurements. Samples were stored at -80°C until required.

2.8.2 Protein extraction from human tissue

Human *post-mortem* frozen samples were lysed at RT in sucrose/SDS buffer (lysis buffer) containing 1% SDS, 0.1 g/mL sucrose,1 mM EDTA, protease inhibitor cocktail at 1:10 (05892791001, Roche), phosphatase inhibitor cocktail at 1:100 (P5726, Sigma) and the serine/cysteine protease inhibitor phenylmethanesulfonyl fluoride at 1:100 (PMSF, P7626, Sigma). Approximately 100 mg of tissue were lysed in 500 µL of sucrose/SDS buffer, added in 3 steps. First, 200 µL of lysis buffer was added and samples were homogenised using polypropylene pellet pestle (Z359947, Sigma) and a pellet pestle motor (Kimble® Kontes, Z359971, Sigma) for 30 s. Then, samples were spun at 3 rpm for 1 min at RT to collect contents and reduce bubbling caused by the SDS. Another 200 µL was added and samples were homogenised again for 10 s. Finally, 100 µL was added and samples were centrifuged at 16 g for 20 min at 16°C. Pellets were discarded and supernatants were collected in a fresh tube, constituting the stock solutions. Stock solutions were

diluted 1:10 in lysis buffer to generate the working concentration sample. Protein concentration of these samples was determined in the same manner as mentioned above for the acute hippocampal slices protein extraction. Samples were aliquoted and stored at -80°C until required.

2.8.3 Western blot

Protein samples (10 µg) were diluted in 6x Laemmli sample buffer to obtain a 1x concentration. For all samples, 10 µg of protein amount were loaded in a 10% polyacrylamide gel. Proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) at constant voltage (90 mV through the stacking gel and 120 mV through the resolving gel) for approximately 1,5 h. 5 µL of protein marker (Color Prestained Protein Standard Broad Range 11–245 kDa, New England Biolabs P7712) with bands of known molecular weight was loaded as a reference. Samples were then transferred into a polyvinylidene difluoride (PVDF) membrane (Immobilon-P, pore size 0.45 µm, IPVH00010, Millipore) by wet electroblotting at constant current (300 mA) for 1 h. A cooling unit was included in the tank to keep temperature low. Membranes were blocked by incubation with 5% skimmed dried milk (Tesco, United Kingdom) in tris-buffered saline-Tween 20 (TBS-T) in constant agitation for 1-2 h at RT. Membranes were then incubated with primary antibodies (see **Table 7**) prepared in 5% milk/TBS-T or 5% bovine serum albumin (BSA)/TBS-T overnight at 4 °C in constant gentle agitation. Next day, membranes were washed with TBS-T for 30 min in constant agitation, changing the TBS-T every 5-10 min. Next, they were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies prepared in 5% milk/TBS-T in constant agitation for 1 hour at RT. Secondary antibodies used in this study include anti-mouse HRP conjugate (1:4000, 12-349, Upstate) and anti-rabbit HRP conjugate (1:4000, 12-348, Millipore). After incubation, the wash step was repeated and membranes were incubated with hydrogen peroxide (H_2O_2) and luminol (1:1 v/v, EZ-ECL, 20-500-120, Biological Industries) for chemiluminescence detection of the antibodies. The chemiluminescent signal was then detected by placing the membrane inside the imaging system (G:Box Chemi XT4, Syngene) and images were taken with GeneSys software (Syngene ©2009-2017, Cambridge, UK). **Figure 2-2** shows a schematic representation of the experimental procedure for human tissue and acute hippocampal slices western blot analysis.

Table 7. Primary antibodies used in this study.

Antibody	Cat. Nº/Supplier	Dilution	Reference
Anti-GSK3β-H76	Santa Cruz (sc-9166)	1:200	(Mitic et al. 2017)
Anti-P-GSK3β	Cell Signal (9336L)	1:1000	(Nagaoka et al. 2015)
Anti-NR1	Upstate (06-31)	1:1000	(Matsuno et al. 2015)
Anti-GluA2	Millipore (MAB397)	1:1000	(Peng et al. 2015; Park et al. 2007)
Anti-mGluR1	ProteinTech (19955-1-AP)	1:1000	(Koochekpour et al. 2012)
Anti-mGluR5	Millipore (AB5675)	1:2000	(Deschwanden et al. 2011)
Anti-mAChR1	Millipore (AB5164)	1:200	(Molina et al. 2014; Takamori et al. 2007)
Anti-Homer 1b/c	Abcam (ab97593)	1:1000	(Matosin et al. 2016)
Anti-PSD95	Cell Signal (3409)	1:1000	(Martineau et al. 2018; Subkhangulova et al. 2018)
Anti-synaptophysin	Cell Signal (4329)	1:1000	(Tiwari et al. 2016)

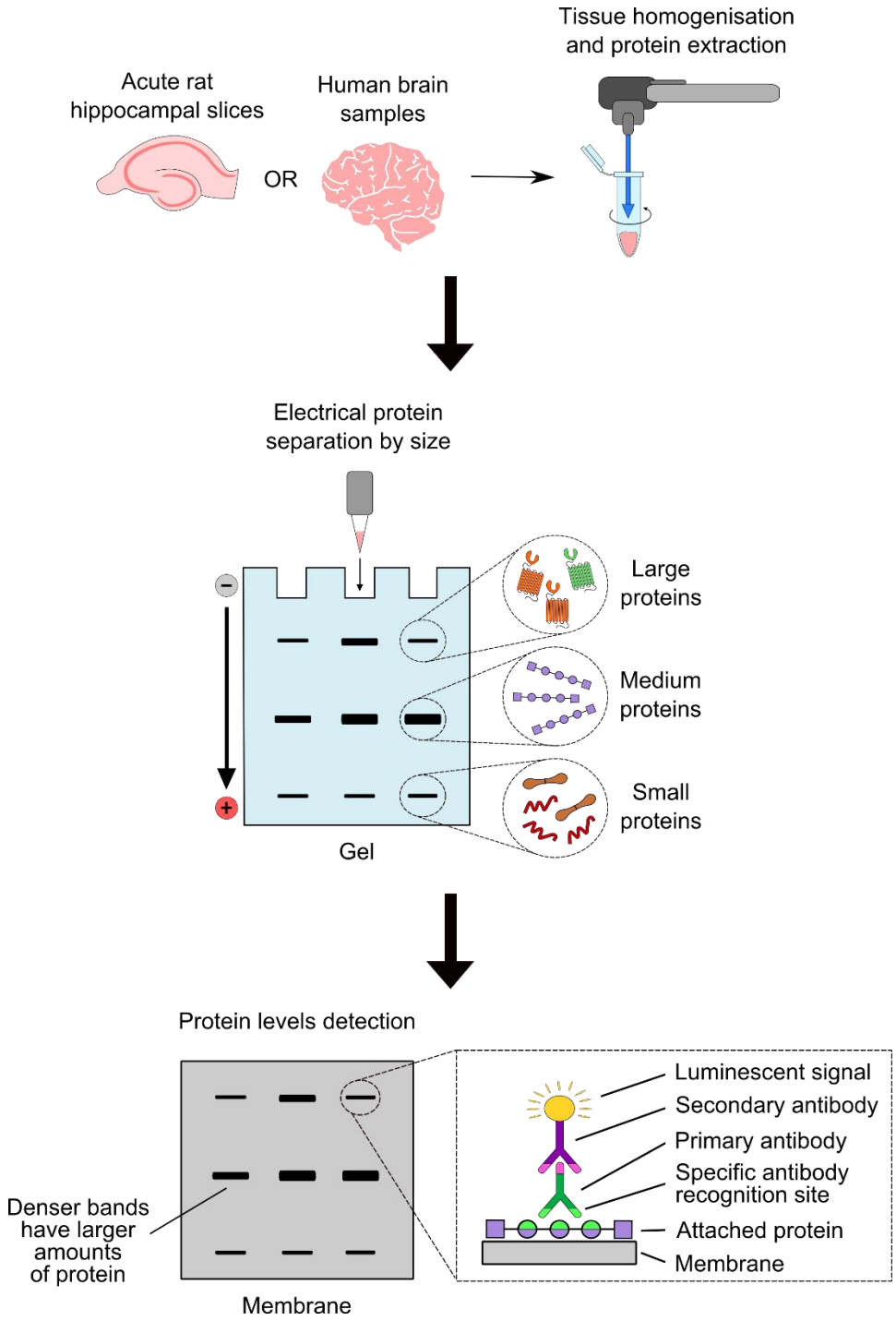


Figure 2-2. Schematic diagram of western blot analysis. Tissue from *post-mortem* human brain samples or acute hippocampal slices was first homogenised. Then, samples were subjected to SDS-PAGE to separate proteins according to size. Finally, proteins were transferred to a PVDF membrane for immunodetection of proteins of interest.

2.8.4 Western blot data analysis and statistics

Optical densities of immune reactive bands were measured using ImageJ software (v1.46r, National Institute of Health, USA). Optical densities of the protein of interest were normalised to β-Actin to account for inaccuracies in gel loading and/or

pipetting. These values were then divided by the average of control experiments and expressed as a percentage of control. In the case of western blot experiments with human samples, due to the heterogeneity of the control samples, data was just normalised to β-Actin.

2.9 Co-immunoprecipitation (Co-IP) assay with human tissue

The following procedures were conducted on ice.

2.9.1 Tissue homogenisation

Human *post-mortem* frozen samples were allowed to thaw on ice prior homogenisation of the tissue. The samples were lysed on ice in Co-IP lysis buffer containing 25 mM Tris (pH 7.6), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, autoclaved double distilled H_2O and protease inhibitor cocktail (05892791001, Roche) at 1:10. Approximately 100 mg of tissue were lysed in 500 µL, added in 3 steps. First, 200 µL of Co-IP buffer was added and samples were homogenised using a polypropylene pellet pestle (Z359947, Sigma) and a pellet pestle motor (Kimble® Kontes, Z359971, Sigma) for 30 s. Next, 200 µL was added and samples were homogenised again for 10 s. Finally, 100 µL was added and the samples were placed on a rotator wheel at 4°C for 30 min. Samples were centrifuged at 10,000 g at 4°C for 10 min. Supernatants were then collected in fresh tubes and the pellets were discarded or stored in case that the presence of the protein of interest in the supernatant was uncertain.

2.9.2 Wash of protein-G agarose beads

Protein-G agarose beads (16-266, Millipore) were resuspended thoroughly before use. A volume of approximately 100 µL of beads was washed three times by adding 1 mL of stock Co-IP buffer (without addition of protease inhibitor cocktail) each time. Tubes were mixed by inversion and gentle flicking to ensure resuspension, beads were spun at 5,000 rpm for 30 s at RT and supernatant was aspirated between each wash. After final wash, beads were resuspended in a volume of Co-IP buffer equivalent to the start volume of beads (100 µL).

2.9.3 Pre-clearing of lysates

This step was performed to reduce non-specific binding of proteins to protein-G agarose beads. A volume of 30 µL of beads was resuspended thoroughly and

added to each sample. Samples were placed on the rotator wheel for 1 h at 4°C. Next, samples were centrifuged for 5 min at 4°C to pellet the beads. This supernatant (pre-cleared lysate, referred here as input) was then transferred into a fresh tube and quantified using the BCA assay as explained above. At this point, inputs could be stored at -20°C for further use.

2.9.4 Pull-down antibody incubation

IP samples were prepared by diluting 500 µg of input in Co-IP buffer up to a volume of 500 µL, leaving the samples at a concentration of 1 µg/µL. One sample was randomly chosen to be incubated with an IgG antibody (Santa Cruz) of the same species as the pull-down antibody, as a negative control. Next, 2 µg of pull-down antibody were added and incubated on the rotator wheel overnight at 4°C.

2.9.5 Protein-G agarose beads incubation

Protein-G agarose beads were resuspended thoroughly by inversion and gentle flicking of the tubes. Next, 30 µL of beads were added to each IP sample, including the IgG control tube. Samples were placed on a rotator wheel for 1 h at 4°C. Samples were spinned at 5,000 rpm for 30 s. Resulting supernatant contained proteins that did not bind to the protein-G agarose beads, hence termed unbound fraction (UB). The unbound fractions were transferred to fresh tubes and stored at -20°C for further use.

2.9.6 Washing steps

Pellets from previous step containing protein-G agarose beads and bound proteins were washed four times. Two first washes were done by adding 1 mL of Co-IP buffer to each sample each time and two last washes by adding 1 mL of 1x tris-buffered saline (TBS). Tubes were mixed by inversion and gentle flicking to ensure resuspension of the beads, spun at 5,000 rpm for 30 s at RT and supernatant was aspirated between each wash. After the final wash, supernatant was carefully removed as much as possible with a pipette to avoid the removal of beads.

2.9.7 Protein elution and gel loading

At this point samples were no longer kept on ice. Proteins were eluted by adding 50 µL of 2x Laemmli sample buffer to each sample. For unbound fractions and input lysates, a 5% of the amount of input used for the antibody incubation was

diluted in 6x or 2x Laemmli sample buffer. Samples were boiled at 95°C for 5 minutes, vortexed to mix and spun at 8,000 rpm for 3 min. Samples were loaded into 10% polyacrylamide gels and western blot was performed as previously described. For IP samples, 20 µL of supernatant was loaded into the gel, carefully avoiding including any beads. Remaining input, unbound fractions and IP samples were stored at -80°C for further use.

2.10 Biolistic transfection of organotypic cultured hippocampal slices

Broadly, biolistic transfection involves the preparation of cartridges containing DNA constructs of interest. These cartridges are then used to transfect cultured hippocampal slices by means of a gene gun that uses compressed helium to transfer the DNA from the cartridge into the slice (**Figure 2-3**).

2.10.1 DNA constructs for biolistic transfection

Cultured hippocampal slices were transfected with short-hairpin RNA (shRNA) against rat tau protein (rTau) cloned in a pGFP-V-RS vector (OriGene Technologies, Md, USA) under the control of the U6 promoter. This vector also contains turbo GFP (tGFP) under the control of the CMV promoter to allow for fluorescent labelling of transfected cells. A mixture of 4 different rat tau constructs (see sequences in **Table 8**) were used in a ratio of 1:1:1:1 to knock-down endogenous rTau (Kimura et al. 2013; Regan et al. 2015). In addition, slices were transfected with one of the following human tau mutants: Wild-type human tau (WT-hTau) (provided by Mandelkow Lab), pseudo-phosphorylated human tau P-hTau or hTau-AT8 (provided by Takashima Lab). These mutants were cloned in pNG2 vectors (derivative of the pET3a, Novagen) and the expression of the mutants was under the control of the AmpR promoter.

Table 8. Rat tau shRNA sequences.

Construct	Sequence
TG710015A/GI740061	AGTCACCGTCTGCCAGTAAGAGCCGCCTA
TG710015B/GI740062	GACACATCTCCACGGCACCTCAGCAACGT
TG710015C/GI740063	ATAGTCTACAAGCCAGTGGACCTGAGCAA
TG710015D/GI740064	AAGGTGACCTCCAAGTGTGGTTCCTTAGG

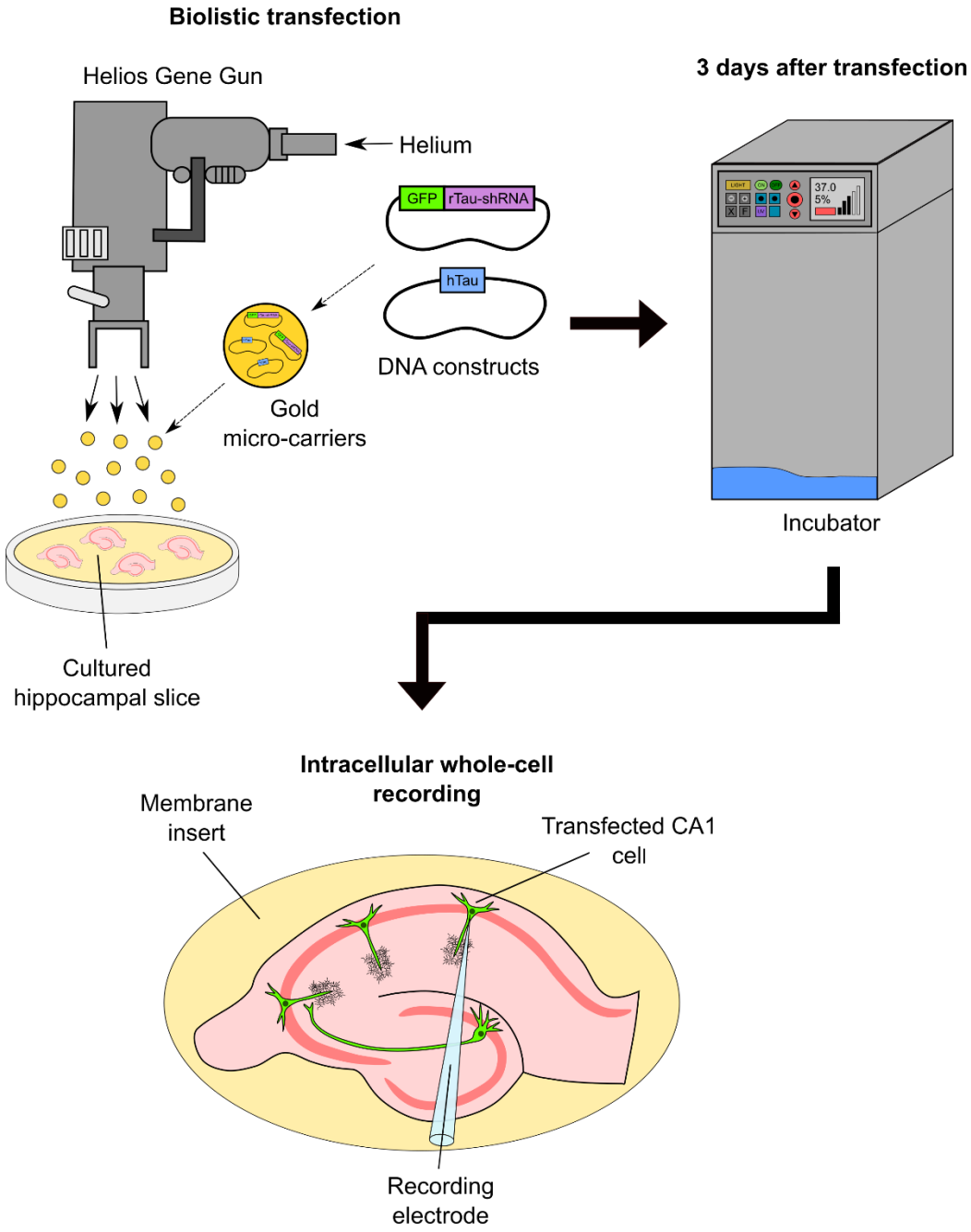


Figure 2-3. Schematic diagram of biolistic transfection and intracellular whole-cell recording experiments in cultured hippocampal slices. Cultured hippocampal slices were biolistically transfected with the Helios Gene Gun. Bullets contained gold microcarriers with the attached DNA constructs of interest. In this example, one plasmid contained the GFP tagged rat tau shRNA and the second plasmid the human tau construct. Cultured hippocampal slices were left in the incubator for 3 days after transfection to allow expression of the constructs. Then, patch clamp was performed to obtain whole-cell recordings from the soma of CA1 neurons in the pyramidal cell layer.

Pseudo-phosphorylated constructs contained serine residues replaced by glutamate residues to mimic constitutive phosphorylation. P-hTau was mutated at S199/S202/T205/T212/S214/S396/S404E residues and hTau-AT8 was mutated at S199/S202/T205 residues.

2.10.2 Precipitation of DNA into gold microcarriers

The total volume of DNA was 100 µl at a concentration of 1 µg/µL. DNA constructs of interest were mixed in a single tube in the following quantities: 15 µg of each rTau-shRNA plasmid (4 plasmids in total) and 40 µg of hTau plasmid of interest (WT-hTau, P-hTau or AT8-hTau). Separately, 100 µl of spermidine (S0266, Sigma) were added to 10 mg of gold microcarriers (165-2264, Bio-Rad) and the mixture was vortexed for 10 seconds and then sonicated for 5 seconds to avoid aggregation of gold microcarriers. DNA was then added to this mixture and vortexed for 5 seconds. Next, 100 µL of 1M calcium chloride was added dropwise to the mixture while vortexing at low speed. Once added, the mixture was vortexed briefly at high speed. The mixture was allowed to precipitate for 10 minutes at RT. A stock solution of polyvinylpyrrolidone (PVP, Bio-Rad) at 20 mg/mL was prepared in 100% ethanol. The solution was diluted with ethanol to obtain PVP working solution at 0.1 mg/mL in a Falcon tube. To ensure that the gold microcarriers remained into the precipitated pellet, the tube was centrifuged in a microcentrifuge for 15 seconds at 2000 g. The resulting supernatant was discarded with a pipette. The pellets were then washed with 1 mL of 100% ethanol, resuspended by gentle flicking of the tube and then spun for 5 seconds in a microcentrifuge and supernatant discarded. This wash step was performed three times. After final wash, supernatant was discarded with a pipette and the pellet was resuspended in 200 µL of PVP working solution. The solution was then transferred in a dropwise manner to the Falcon tube containing the remaining PVP working solution. The microcentrifuge tube was washed a few times with PVP working solution to ensure all particles were collected. The Falcon tube was then vortexed to thoroughly mix the solution.

2.10.3 Loading the DNA/microcarrier suspension into tefzel tubing: Tubing Prep Station

First, tefzel tubing (BioRad Laboratories Ltd., Hemel Hempstead, UK) was secured in the Tubing Prep Station (BioRad Laboratories Ltd., Hemel Hempstead, UK) and dried for at least 30 minutes by perfusing nitrogen through it (0.3-0.4 litres per minute, LPM). After this time, nitrogen perfusion was turned off. Immediately before loading, the DNA/microcarrier solution was vortexed to ensure an even suspension of the mixture. A syringe connected to the end of the tefzel tubing was used to slowly pass the solution through the tubing. Pressure in the syringe was held for 2 minutes to allow settling of the microcarriers at the bottom of the tubing. Next, the

loading solution was slowly aspirated back into the syringe. Tubing was then turned 180° and left for 30 seconds. Next, the Tubing Prep Station was set up for constant and slow rotation for one minute. After this, perfusion of nitrogen at a flow of 0.3-0.4 LPM was started to dry the tubing while still rotating, for 5 minutes, and then turned off.

2.10.4 Preparation of cartridges

Tubing was visually inspected to verify that microcarriers were evenly distributed over the length of the tubing and to avoid clumped or bare sections. Otherwise some cartridges may not contain microcarriers which will affect transfection. Tubing was then inserted into the Tubing Cutter (BioRad Laboratories Ltd., Hemel Hempstead, UK) to cut the tubing into ~1.3 cm pieces (cartridges). Cartridges were then stored in a screw-cap tube containing drierite stones (CamLab, Cambridge, UK) to prevent moisture build up and stored at -20°C until required.

2.10.5 Biolistic transfection

The Helios Gene Gun (BioRad Laboratories Ltd., Hemel Hempstead, UK) was used per manufacturer's instructions. Briefly, helium was supplied into the gene gun at a pressure of 180 pounds per square (PSI). This pressure was optimal to propel the microcarriers into the slices without damaging the tissue. Cartridges were loaded into the cartridge holder which was locked into place in the Helios Gene Gun. The barrel liner of the Helios Gene Gun was positioned ~2 cm above of the well containing the membrane to be transfected and in a perpendicular position to the base of the 6-well plate. Finally, the safety button was engaged, and the firing trigger pressed to fire the microcarriers into the slices. Cultured slices were transfected at 3 DIV and electrophysiological recordings were performed at 3-5 days after transfection (7-12 DIV). Identification of GFP-transfected cells was achieved by using an LED excitation light at a wavelength of 470 nm (**Figure 2-4**).

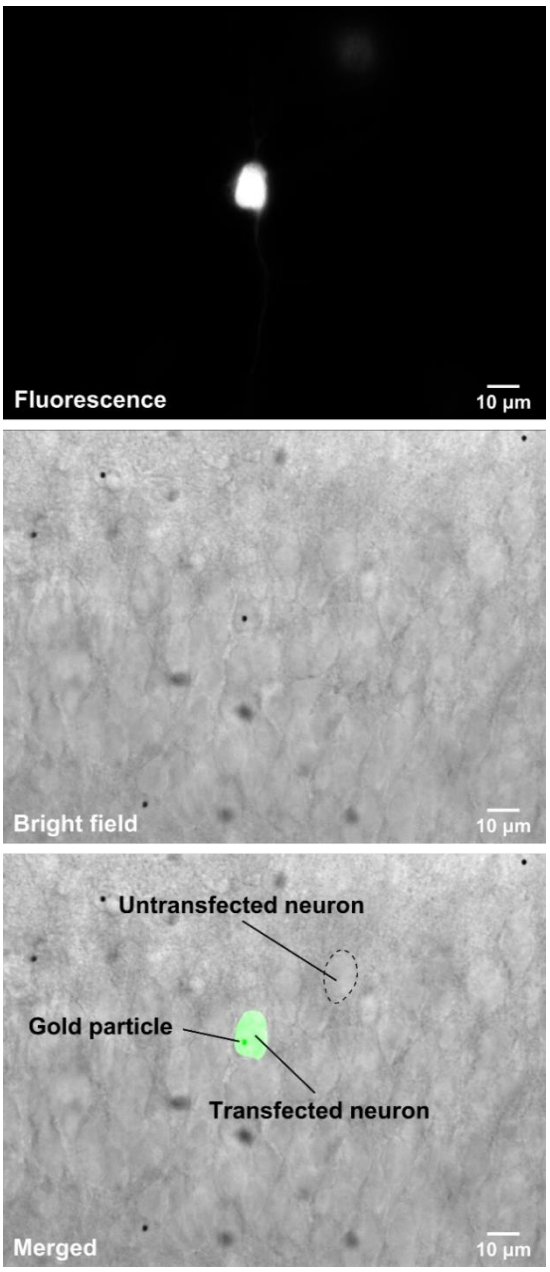


Figure 2-4. Transfected neuron in a cultured hippocampal slice. Image taken at 40x magnification showing a neuron transfected with a GFP-tagged construct surrounded by untransfected neurons. The gold microcarrier is usually visualised as a black dot on the soma of the cell.

2.11 *In vitro* electrophysiological recordings in rat slices

2.11.1 Equipment set-up

The slice was submerged into the recording chamber and immobilized by placing an in-house constructed nylon mesh net on top of it. The aCSF was delivered through PTFE microbore tubing (0.022 inches inner diameter x 0.042 inches outer diameter, WZ-06417-21, Cole Parmer, Cambridgeshire, UK) which was inserted

into a heated perfusion tube system (HPT, ALA Scientific Instruments Inc., USA) controlled by a TC-10 temperature control system (npi electronic GmbH, Tamm, Germany). This served to maintain the aCSF within the recording chamber at 29–31°C. A glass bottle containing aCSF bubbled with 95% O_2/5% CO_2 was submerged into a heated water bath (Clifton, Nickel-electro, Weston-super-Mare, UK) to keep the aCSF at ~28 °C. The recording chamber was constantly perfused with aCSF at a flow rate ~ 3 mL per min. This was achieved by pumping the aCSF into the recording chamber through fine bore polythene tubing (1.67 mm inner diameter, 2.42 mm outer diameter, 800/100/460, Portex Ltd., Kent, UK) by a peristaltic pump (Sci-Q 323, Watson-Marlow Ltd., Falmouth, UK). aCSF was removed via a suction needle into a waste bottle connected to a vacuum pump (Dymax 5, Charles Austen Pumps Ltd, Surrey, UK).

An anodized aluminium platform (P-1, 64-0277, Warner Instruments, CT, USA) provided clamping between a polycarbonate recording chamber (RC-26G, Warner Instruments, CT, USA) and a glass coverslip base (22 x 40 mm, 64-0707, Warner Instruments, CT, USA). A water tight seal was obtained by applying vacuum grease between the bottom of the platform and the edges of the glass coverslip. This was mounted upon a movable top plate (Scientifca, Sussex, UK) that allowed the movement of the recording chamber and therefore the slice in the X and Y axis, so it could be centred in the microscope field of view. The microscope and movable top plate were fixed to an air table (IsoStationTM, Newport, UK) to minimize vibration from external sources. The air table was filled with compressed air by an air compressor (JunAir 3-4, 1109020, MI, USA). To prevent interferences from external electrical fields, the air table and equipment mounted on it were covered by an in-house built Faraday cage.

Slices were visualised with a fixed-stage upright microscope Olympus BX51WI (Olympus, Tokyo, Japan) and an attached charged-coupled device KP-M1AP camera (Hitachi, Tokyo, Japan). GFP fluorescence signals were visualised with an LED illumination system (CoolLED pE-100, Andover, UK) that permitted illumination of slices at a wavelength of 470 nm.

2.11.2 Recording and stimulating electrodes

Recording pipettes were made from borosilicate glass capillary (standard wall, 1.5 mm outer diameter x 0.86 mm inner diameter x 100 mm long, Harvard Apparatus,

Kent, UK) pulled with a P-1000 Flaming/Brown micropipette puller (Sutter Instrument Co., CA, USA) to a resistance of 5-7 MΩ. They were back-filled with the appropriate filling solution, depending on the type of recording (see sections **2.11.3** and **2.11.4**), and then secured in an electrode holder (QSW-T15P, Warner Instruments, CT, USA), which was connected to a headstage (CV-203BU, Molecular Devices, CA, USA). The movement of the recording pipette was precisely controlled with an electronic PatchStar micromanipulator (Scientifica, Uckfield, UK), which allowed fine movements in the X, Y and Z axes, permitting precise position of the recording pipette on the slice.

The recording electrode was a silver wire (99.9% purity, 0.20 mm diameter, Advent Research Materials, Oxford, UK) coated with silver-chloride (AgCl). This coating allows for a smooth flow of current from the intracellular or extracellular space to the electrode. Importantly, current must be transformed from a flow of ions in the solution to a flow of electrons in the recording electrode. However, current crossing the interface between the filling solution and the silver wire can suffer signal distortion or loss. The Ag-AgCl coating prevents this by providing a reversible reaction that ensures the bidirectional flow of electrons. Electrons flowing from the wire to the Ag-AgCl interface reduce the silver (Ag^+) back to silver atom (Ag), releasing a chloride ion (Cl^-) that becomes hydrated and enters the solution. If electrons flow from the solution to the Ag wire, the Ag loses one electron becoming Ag^+, which forms insoluble AgCl with a nearby Cl^- staying within the coating layer (Dong & Graziane 2016). The current through the recording electrode was transmitted to the headstage through a gold pin connected to it. A reference electrode coated in Ag-AgCl to the point that was in contact with the bath solution was submerged in the bath of the recording chamber and also connected to the headstage through a gold pin.

Bipolar stimulating electrodes were made of two inter-coiled nickel 80/chromium 20 wires (0.050 mm diameter, Advent Research Materials, Oxford, UK). The stimulating electrode was passed through a glass capillary which was placed in a manual micromanipulator (LBM7, Scientifica, Uckfield, UK) that allowed fine positioning of the stimulating electrodes on the slice. Each stimulating electrode was connected to an isolated stimulating box unit (DS2A-Mk.II, Digitimer Ltd., Welwyn Garden City, UK).

2.11.3 Extracellular field recordings

One acute hippocampal slice was placed in the recording chamber and perfused with aCSF which comprised 124 mM NaCl, 3 mM KCl, 26 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 2 mM $CaCl_2$, 1 mM $MgSO_4$ and 10 mM D-glucose and bubbled with 95% O_2/5% CO_2. The recording pipette was back-filled with 3 M NaCl filling solution and was placed on the *stratum radiatum* of the CA1 region of the slice. Here is where the axon terminals of Schaffer collaterals coming from the CA3 region establish synaptic connections with the apical dendrites of CA1 pyramidal neurons (**Figure 2-5**). One stimulating electrode was placed on the Schaffer collaterals to deliver high-frequency stimulation (HFS) on this pathway, therefore termed HFS input. Another stimulation electrode was placed on the subiculum region, between the CA1 and entorhinal cortex, to function as control (i.e. non-tetanised) input. As a result of the stimulation delivered to the Schaffer collaterals, extracellular field excitatory post-synaptic potentials (fEPSPs) were evoked in the CA1 region and recorded throughout the experiment. Therefore, fEPSPs are the population synaptic response which arises from the synchronous and localised currents generated as a result of synaptic stimulation of pyramidal CA1 cells (Dong & Graziane 2016).

The response obtained after stimulation includes components other than the fEPSPs. Immediately after stimulation, a stimulus artefact is generated as a result of the voltage pulse applied (**Figure 2-6A**). Subsequently, it is very common to see a fiber volley which is recorded by the extracellular electrode as a negative potential. This represents the action potential firing of axons within Schaffer collaterals. These activate the release of neurotransmitter at the presynaptic terminal and produce an EPSP in the dendrites of CA1 neurons. Consequently, the more fibers activated with higher stimulus intensity will result in greater fEPSPs. Importantly, this is not due to an enhancement of the fEPSPs at individual synapses, as it would happen in the case of LTP, for example. This means that the amplitude of the fiber volley is proportional to the number of presynaptic fibers that are active within the Schaffer collaterals and that changes in the fiber volley amplitude result in changes in the fEPSP. Therefore, only recordings with no change in fiber volley amplitude were counted for analysis. Finally, some fEPSP can be contaminated by population spikes. This represents action potential firing in the somata of a population of neurons. A population spike is caused by the antidromic propagation of dendritic EPSPs from the dendrites to the soma, which

can trigger an action potential when the stimulus strength is high enough. The population spike is recorded as positive potential that can limit the amplitude of the fEPSPs, but the slope generally remains unaffected.

At the electrophysiological level, changes in synaptic efficacy are reflected by changes in the fEPSP size (for instance, LTP is defined as a sustained increase in the fEPSP size). Accordingly, the slope of the fEPSP was used as a measure of synaptic efficacy to detect changes in fEPSPs size. The measurement of the slope was preferred to the amplitude because the latter is often contaminated with population spikes (explained above), population IPSPs and polysynaptic responses (arising from synaptic events occurring at different time points following an evoked stimulus).

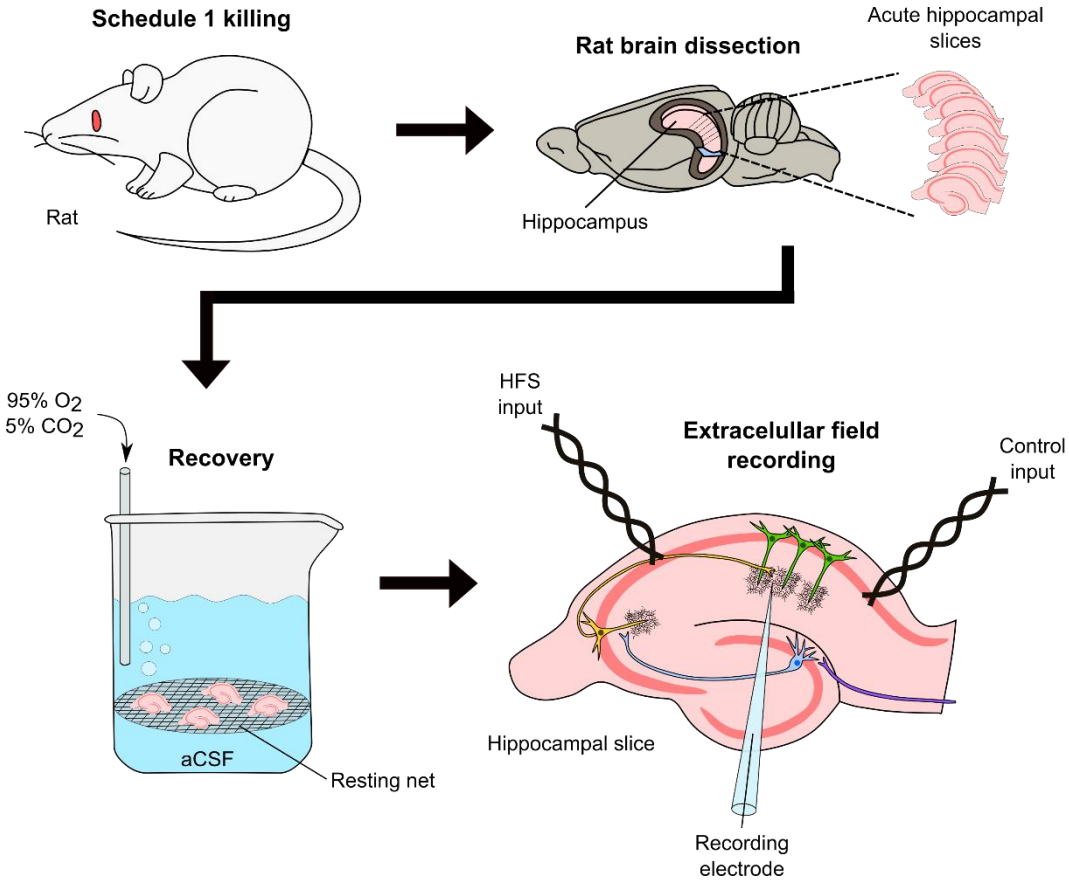


Figure 2-5. Schematic diagram of extracellular field recoding experiments in acute hippocampal slices. P24-30 Wistar rats were killed by cervical dislocation and brain dissection was performed to prepare acute hippocampal slices. Dotted lines roughly indicate the planes for cutting the hippocampus. Acute hippocampal slices were allowed to recover for 1 h in aCSF at room temperature prior starting the recordings. Extracellular field recordings were obtained from the *stratum radiatum* of the CA1 region. One stimulation electrode was placed on the Schaffer collaterals (HFS input) and another one on the subiculum area (control input).

Stimulus intensity was empirically determined as the intensity that yielded 40% of the maximal fEPSP amplitude, but never higher than 13 V to avoid rundown of responses due to overstimulation and contamination by non-synaptic signals. Stimuli at constant voltage were delivered alternately to the two electrodes (HFS and control input) every 15 seconds (0.033 Hz). This stimulation will be referred as basal stimulation. A stable baseline, usually between 10-30 min depending on the type of experiment, was first obtained before performing further experimental manipulations. In LTP experiments, after recording a 30 min baseline, tetanic stimulation was delivered to only the electrode placed on the Schaffer collaterals. This protocol consisted in 2 trains of 100 Hz, i.e. each train comprises 100 pulses in 1 second, with an interval of 30 seconds between trains. Then, basal stimulation was delivered for at least 60 min after tetanus.

Each sweep had a duration of 50 milliseconds (ms) and comprised 500 voltage measurements (i.e. samples) in mV, with a sample interval of 0.1 ms. The slope start and end times were manually defined as milliseconds after stimulation for each experiment. Then, a linear regression line was plotted through the voltage data points falling in between those defined time points. The slope value (mV/ms) for each response was generated from the regression line. Slope values were averaged from 4 consecutive responses and were plotted as a single data value.

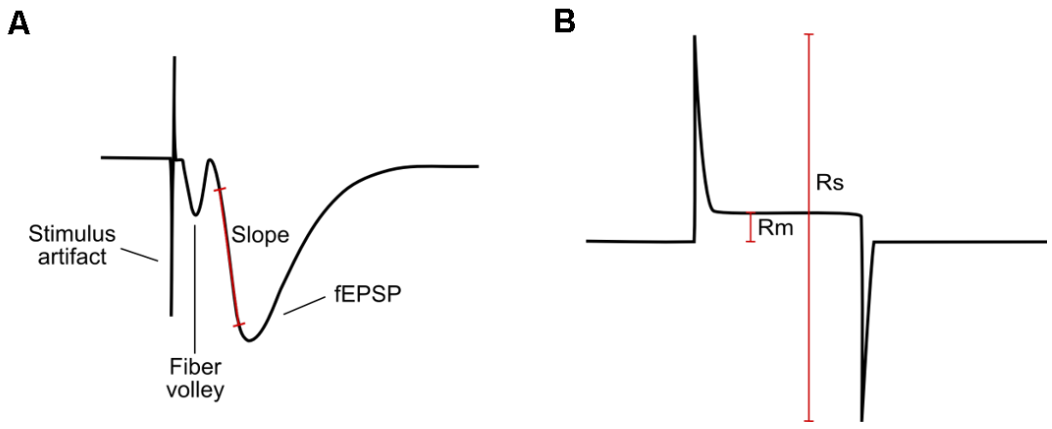


Figure 2-6. Measured parameters in electrophysiological recordings. (A) Example fEPSP trace from extracellular field recordings showing stimulus artefact, fiber volley and slope measurement. **(B)** Series resistance (Rs) and membrane resistance (Rm) were measured in response to a voltage pulse (10 mV, 100 ms) in intracellular whole-cell recordings.

2.11.4 Intracellular whole-cell patch clamp recordings

These experiments were performed in cultured hippocampal slices bound to membrane inserts. For each experiment, a section of the insert membrane containing one slice was cut with the aid of a blade and forceps and it was placed in the recording chamber. The slice was perfused with aCSF comprising: 119 mM NaCl, 2.5 mM KCl, 26 mM $NaHCO_3$, 1 mM NaH_2PO_4, 11 mM D-glucose, 4 mM $CaCl_2$, 4 mM $MgCl_2$, 0.02 mM picrotoxin, 0.002 mM 2-chloroadenosine, 10 µM MK-801, 500 nM tetrodotoxin citrate (TTX) and gassed with 5% CO_2/95% O_2. The recording pipette was filled with potassium-gluconate filling solution containing: 10 mM HEPES, 135 mM potassium-gluconate, 0.5 mM EGTA, 2 mM Mg^{2+}-ATP, 0.3 mM Na^{+}-GTP, 8 mM NaCl (275-290 mOsm, pH 7.2).

The CA1 region was visually located under the 5x objective and then cells within the pyramidal cell layer were located under the 40x objective (**Figure 2-3**). Positive pressure was applied to the recording pipette via a pneumatic system consisting on syringe attached to a piece of Tygon tubing which in turn was tightly connected to an aperture (port) in the electrode holder. The 40x objective was separated from the preparation and the recording pipette was placed inside the bath. The resistance of the recording pipette tip was checked with the amplifier on “track” mode and “seal test” on, by applying a rectangular voltage pulse of +5 mV and measuring the resulting square pulse of current. According to Ohm’s law (V = I x R), the amplitude of this current is inversely proportional to the resistance of the pipette tip. Only recording pipettes with a tip resistance between 5–7 MΩ were used. Next, the recording pipette was lowered to the cell layer slowly together with the objective. With the target cell in focus, the recording pipette was advanced to the surface of the cell by using the micromanipulator. As the pipette approached the cell soma, the positive pressure pushed the membrane generating the formation of a dimple. At this point, positive pressure was released to induce a suction effect that caused the cell membrane to form a seal around the pipette tip. The formation of this seal was evident by an increase in the monitored pipette resistance (decrease of the current through the pipette tip). Amplifier settings were immediately changed to voltage clamp, “V-Clamp”, and a negative holding potential was applied, initially at -30 mV that was slowly increased until reaching -70 mV. The formation of a GigaOhm (GΩ) seal was evident by the reduction of the current pulse to around -20 pA. In this cell-attached mode, the external command of the amplifier was switched on and the capacitive transients of the pipette could

be seen in the current pulse. These capacitive transients arise as a result of the capacitive properties of the pipette that generates charge separation across the glass. They represent the removal of anions (in this case mainly chloride ions since the use of Ag-AgCl electrodes and a positive voltage pulse) from the intracellular space. Capacitive currents were compensated with the amplifier to avoid distortion of the current of interest. To break into the cell, a sharp, quick negative pressure was applied by gentle suction with the mouth into the syringe. This was generally sufficient to enter whole-cell configuration and the characteristic capacitive transients of the membrane were monitored (**Figure 2-6B**).

Once in whole-cell configuration, the resting membrane potential (RPM) of the cell was checked and cells with RPM below -50 mV were discarded. The holding current (DC), which is the current applied to the cell to hold the membrane potential at the commanded voltage of -70 mV, was recorded throughout the experiment. Additionally, a square voltage pulse of 10 mV during 100 ms was applied to monitor series resistance (R_s) and membrane resistance (R_m). R_s describes the pipette resistance and the access resistance once in whole-cell configuration, as they are both in series with the pipette voltage in the equivalent electrical circuit. The access resistance refers to the resistance between the membrane of the cell and the pipette tip. Since pipette resistance does not change during an electrophysiological recording, changes in the series resistance are caused by changes in the access to the cell. Therefore, series resistance was used to monitor the quality of the patch and only experiments with a R_s less than 23 MΩ and changes less than 20% of initial R_s were accepted. R_m, also termed input resistance, was used to monitor the condition of the cell. Only cells with a R_m bigger than 80 MΩ were considered healthy and therefore accepted. Each sweep had a duration of 200 ms and comprised 10,000 voltage measurements (i.e. samples) in mV, with a sample interval of 0.05 ms.

2.11.5 Electrophysiological data acquisition, analysis and statistics

The headstage was connected to an amplifier (Axopatch 200B, Axon Instruments Inc., Union City CA, USA) for signal recording. In turn, the amplifier was connected to a digital-to-analogue/analogue-to-digital (DAC-ADC) BNC 2120 board (National Instruments, Berkshire, UK) connected to a M Series data acquisition device (DAQ) device board (National Instruments, Berkshire, UK) in the computer. The

amplifier low-pass filter was set to filter signals at 5 kHz and to amplify them 5x (output gain).

Data was analysed online using WinLTP (WinLTP Ltd., University of Bristol, Bristol, UK, 1991-2018) software. This software allowed for automated response recording and stimulus delivery throughout the experiment as well as real-time monitoring of the recorded synaptic responses and associated parameters. SigmaPlot 13.0 software (Systat Software Inc., San Jose, CA, USA) was used for statistical analysis and to graph the data.

For field recording experiments, data were normalised to the pre-conditioning baseline and expressed as a percentage of the same baseline (i.e. each slope value was divided by the average slope value of the baseline and multiplied by 100). For each experimental condition, i.e. control or treatment, data from one slice per rat were analysed (n = number of slices = number of rats). Normalised data were then pooled across slices for each experimental condition to obtain a mean slope value ± standard error of the mean (S.E.M) for each time point, which were plotted in the graphs shown. Statistical significance ($p < 0.05$) of the pooled data was tested using paired or unpaired two-tailed Student t-test as appropriate. For LTP experiments, unpaired two-tailed t-test was performed between the group of means generated by averaging slope values at 5 time points after HFS (86, 88, 90, 92, 94 minutes) for the control vs. the group of means for the HFS input. When appropriate, the HFS inputs from two different conditions (i.e. control vs. treatment) were also statistically compared in the same manner. For baseline experiments, unpaired two-tailed t-test was performed between the population of means corresponding to minutes 36, 38, 40 (in 40 minutes recordings) or minutes 72, 74, 76, 78, 80 (in 80 minutes recordings) between control and treated slices.

For whole-cell patch clamp experiments, data was normalised to the 10 minutes baseline (i.e. each DC value was subtracted to the average of the baseline). Therefore, data shown in graphs represents the change in holding current with respect to the baseline. Then, the maximum holding current value for each experiment (i.e. slice) was taken and values were pooled across slices within the same condition. Statistical significance ($p < 0.05$) of the pooled data was tested using unpaired two-tailed Student t-test or Mann-Whitney rank sum test if data were not normally distributed.

2.12 Pharmacological agents and treatments

Pharmacological agents were bath applied by direct addition to the perfused buffer or incubation solution to obtain the concentration of interest. For control experiments, solvents at the concentration used to prepare the stock aliquots were applied in the same manner.

All pharmacological agents used in this study are listed in **Table 9**, which summarizes information regarding mode of action of each agent, suppliers and working concentrations used. For control experiments, solvents at the concentration used to prepare stock solutions were used. Stock solutions were aliquoted and kept at -20°C.

Table 9. Pharmacological agents used in this study.

Name	Abbreviation	Mode of action	Supplier	Concentration used (µM)
(R,S)-3,5-Dihydroxyphenylglycine	DHPG	Group I mGluR agonist	Abcam (ab120020)	20, 10, 50
(R,S)-2-Amino-2-(2-chloro-5-hydroxyphenyl) acetic acid	CHPG	mGluR5 agonist	Abcam (ab120039)	1, 10, 50, 100, 500
(3,4-Dihydro-2H-pyrano[2,3-b] quinolin-7-yl)-(cis-4-methoxycyclohexyl)-methanone	JNJ	mGluR1 non-competitive antagonist	HelloBio (HB0348)	1
(2-Hydroxyethyl) trimethylammonium chloride carbamate (carbachol)	CCh	Cholinergic agonist	Sigma-Aldrich (C4382)	10, 25, 50, 100, 200
(+) MK-801 maleate	MK-801	non-competitive NMDA receptor antagonist	HelloBio (HB0004)	10
Picrotoxin	Picrotoxin	GABAA receptor antagonist	Abcam, (ab120315)	20
Tetrodotoxin citrate	TTX	voltage-gated Na+ channel blocker	HelloBio HB1035	0.5
2-chloroadenosine	2-CADO	adenosine receptor agonist	HelloBio, HB2844	2

In electrophysiology experiments, pharmacological agents used during the recordings were bath applied by direct addition to the perfused aCSF to obtain the concentration of interest. For incubation of pharmacological agents with slices prior

to recordings, an incubation chamber comprised of a nylon mesh net fitted into a Petri dish (35 x 10 mm, Cellstar 627 160) was filled with 5 mL of aCSF and bubbled with 95% O_2/5% CO_2. The pharmacological agent was added to the aCSF and left for 5 min to reach an equilibrated concentration on the solution. Then a slice was placed in the incubation chamber with a glass pipette, avoiding altering the concentration reached in the chamber by not letting aCSF from the pipette going into the solution. In calcium imaging experiments, pharmacological agents were also bath applied.

Chapter 3 Effect of mGluR5 activation on synaptic plasticity

3.1 Introduction

3.2 Results

3.2.1 mGluR5 activation does not affect baseline recording or LTP induction

3.2.2 Selective mGluR5 activation does not affect CCh-induced depression

3.2.3 Effects of transient agonist activation of mGluRs on mAChR-mediated calcium increase

3.2.4 mGluR5 protein expression in acute hippocampal slices

3.2.5 Agonist activation of mGluRs does not affect GSK3β activation

3.3 Discussion

3.3.1 Activation of mGluR5: what makes it detrimental?

3.3.2 mGluRs and mAChRs interplay

3.3.3 mGluRs and GSK3β

3.3.4 Summary

3.1 Introduction

Given the strong deficits in memory exhibited in AD patients, together with the evidence supporting synaptic plasticity as the physiological foundation for such cognitive processes, the disruption of synaptic plasticity has been widely studied in the context of the disease (Jang & Chung 2016; Skaper et al. 2017). The most consistent argument for this concept is the evidence showing that AD-related neuropathology, namely Aβ and tau, is associated with deficits in synaptic function and plasticity.

The ways in which Aβ disrupts synaptic function are numerous but one well-established finding that Aβ reduces glutamatergic synaptic transmission (Chapman et al. 1999; Walsh et al. 2002) by decreasing the number of surface AMPARs and NMDARs (Hsieh et al. 2006; Shankar et al. 2007). This occurs by Aβ-induced activation of LTD mechanisms that include the phosphorylation of GluA2 subunit of AMPARs and the actions of calcineurin and p38 MAP kinase activity (Hsieh et al. 2006; Shankar et al. 2007). This leads to endocytosis of AMPARs which in turns induces NMDARs-endocytosis and it seems to underlie the loss of dendritic spines (Hsieh et al. 2006). In addition, this has been proposed as a mechanism by which Aβ impairs LTP (Cleary et al. 2005; Walsh et al. 2002) and enhances LTD, causing the subsequent synaptic loss that may contribute to memory deficits (Li et al. 2009; Hsieh et al. 2006). These effects are thought to be mediated by oligomeric forms of Aβ (Selkoe 2008; Shankar et al. 2008; Tomiyama et al. 2010), the presence of which correlates better than Aβ plaque load with cognitive impairments in animal models of AD (Mucke et al. 2000) and AD patients (Näslund et al. 2000; McLean et al. 1999). Because synapses are the sites where plasticity manifests, some of the Aβ-mediated effects on synaptic plasticity may occur at the dendritic level. Consistent with this, Aβ acts on dendrites to reduce spine numbers and plasticity (Wei et al. 2010). Part of these effects may be mediated by Aβ preferential binding at postsynaptic sites (Lacor et al. 2004; Lacor et al. 2007) and multiple Aβ receptors have been proposed to mediate Aβ synaptotoxicity (Kam et al. 2014), including mGluRs (Um et al. 2013; Renner et al. 2010). Through the binding to these receptors, or independently, Aβ is able to trigger dysregulation of dendritic ion channels that modulate neuronal excitability (Cochran et al. 2014). For example, Aβ can induce phosphorylation of L-voltage sensitive calcium channels through the activation of mitogen-activated protein (MAP) kinase (Ekinci et al. 1999) and increase calcium influx, generating

excitotoxicity in neuronal cultures (Ho et al. 2001; Ueda et al. 1997). This is in line with the finding that Aβ-induced calcium dysregulation is a major component of synaptic dysfunction (Wang et al. 2017). In addition, excessive Aβ modifies dendritic excitability by disrupting the function of potassium (Ye et al. 2003; Zhang & Yang 2006) and probably sodium channels (Verret et al. 2012).

Not only Aβ-mediated pathology but tau pathology can also affect synaptic plasticity mechanisms. Illustrating this, a transgenic mice model of pathological tau aggregation displays strong deficits in LTP and memory (Sydow et al. 2011) and administration of tau to hippocampal slices and mice impairs LTP and memory, respectively (Fá et al. 2016). These deficits can be rescued by removing the expression of the toxic tau mutant (Sydow et al. 2011), providing evidence for an involvement of tau-mediated pathology in the disruption of synaptic plasticity. Similarly to the case of Aβ, some of these effects occur at the dendrites, where tau is thought to mislocalise at early stages of AD pathology (Zempel et al. 2010; Braak et al. 1994; Braak et al. 2011), potentially as a result of tau phosphorylation (Hoover et al. 2010). Additionally, tau can be phosphorylated at the dendrites (Jin et al. 2011; Zempel et al. 2010), a modification that is required for the Aβ-induced synaptotoxicity and dendritic spine deficits (Gu et al. 2013; Mairet-Coello et al. 2013; Yu et al. 2012). Dendritic tau is often in the form of soluble tau and it has been shown that this form has a prominent pathological role (Cochran et al. 2014). This role involves tau-dependent trafficking of Fyn kinase to the synapse (Ittner et al. 2010) where it potentiates NMDARs excitotoxicity by phosphorylating the NR2B subunit of this receptor (Nakazawa et al. 2001; Rong et al. 2001). In addition, it has been proposed that tau disrupts synaptic function by mediating aberrant scaffolding of the postsynaptic density (Chabrier et al. 2012; Ittner et al. 2010; Mondragón-Rodríguez et al. 2012).

These studies highlight the complexity of Aβ- and tau-induced synaptic dysfunction and the necessity of identifying molecular targets that mediate its detrimental effects. Over the past years, research has brought to light mGluR5 as one such target (Kumar et al. 2015; Piers et al. 2012). As outlined in **Chapter 1**, mGluR5 is a GPCR located in the postsynaptic terminal, preferentially to perisynaptic and extrasynaptic locations (Conn & Pin 1997; Shigemoto et al. 1993; Romano et al. 1995; Lujan et al. 1996; Lopez-Bendito et al. 2002) where it can regulate and mediate synaptic transmission. The role of mGluR5 at glutamatergic synapses also

includes the capacity to mediate long-lasting forms of synaptic plasticity including LTD and LTP (Lüscher & Huber 2010). For instance, activation of mGluR5 can result in the induction of LTD in the CA1 region of the hippocampus (Bolshakov & Siegelbaum 1994; Huber et al. 2000; Kemp & Bashir 2001; Manahan-Vaughan 1997; Volk et al. 2007; Oliet et al. 1997). Importantly, this role of mGluR5 in mediating synaptic plasticity may be related to the role of the receptor in memory. This is supported by *in vivo* studies where pharmacological blockade or genetic knock out of mGluR5 result in impaired spatial learning and LTP in the hippocampus (Lu et al. 1997; Balschun et al. 1999; Balschun & Wetzel 2002; Naie & Manahan-Vaughan 2004; Manahan-Vaughan & Braunewell 2005), implying the requirement of mGluR5 for memory processes.

Considering the physiological synaptic functions of mGluR5, it is not surprising that dysregulation of its expression or function in disease may result in disruption of synaptic function. Indeed, there is strong evidence to support the emerging concept that aberrant mGluR5 activation may mediate Aβ-related pathology in AD (Kumar et al. 2015; Wang 2004; Hu et al. 2014; Um et al. 2013; Hamilton et al. 2014; Renner et al. 2010; Haas et al. 2016). First, studies have shown that the enhancement of LTD (Hsieh et al. 2006; Li et al. 2009) and suppression of LTP (Walsh et al. 2002; Shankar et al. 2008; Jo et al. 2011) triggered by Aβo can be reproduced by activating mGluR5 with agonists but also inhibited by blocking the receptor with antagonists (Wang 2004; Shankar et al. 2008; Hu et al. 2014; Rammes et al. 2011). Second, pharmacological blockade or genetic deletion of mGluR5 reversed the Aβ-induced deficits in learning, memory and spine density (Um et al. 2013; Hamilton et al. 2016; Hamilton et al. 2014). Third, it has been reported that mGluR5 antagonists protect from NMDAR-induced excitotoxic degeneration (Kingston et al. 1999; O'Leary et al. 2000; Movsesyan et al. 2001). This is relevant since Aβ-mediated dysregulation of NMDARs is thought to be a mechanism in the pathology of AD (Texidó et al. 2011; Miguel-Hidalgo et al. 2002; Molnár et al. 2004; Li et al. 2011). According to this hypothesis, antagonism of mGluR5 protects against Aβ-induced toxicity in cortical cultures (Bruno et al. 2000) and also in primary hippocampal neurons from double transgenic mice models of Parkinson's disease and AD, in which lentiviral transfection of mGluR5 in the hippocampus results in neurodegeneration (Overk et al. 2014).

Although a central role of mGluR5 in Aβ-like pathological processes is undeniable, the mechanisms by which it becomes detrimental for neuronal function have not been fully characterised. To understand such mechanisms, two important questions must be considered. First, whether these mechanisms involve mGluR5-mediated regulation of other key molecules in AD. Second, whether this regulation occurs only in pathological conditions or conversely arises from physiological interactions that are enhanced or reduced in pathology.

A critical effect of AD pathology on synaptic function is the impairment of cholinergic transmission, due to the depletion of cholinergic neurons in the basal forebrain (Whitehouse et al. 1982) and their projection to the hippocampus and cerebral cortex (Davies & Maloney 1976; Parent et al. 2013) (see **Chapter 1**). Further supporting this, mAChR1 levels are reduced in brains of AD patients (Shiozaki et al. 2001), which may indicate that reductions in mAChRs function are involved in the pathology of AD. Supporting this, agonism of these receptors rescued impairments in hippocampal-dependent memory in an AD mouse model, indicating that hypofunction of mAChRs negatively affects cognitive processes (Caccamo et al. 2006). In addition, mAChRs deletion results in disruption of LTP and cognitive performance in behavioural tasks (Anagnostaras et al. 2003). Furthermore, genetic deletion or inhibition of mAChR1 results in exacerbation of Aβ and tau pathologies *in vivo* (Davis et al. 2010; Caccamo et al. 2006) whereas its activation down-regulates Aβ production (Hung et al. 1993; Fisher et al. 2003). Therefore, mAChR is a key molecule in the pathology of AD and understanding their regulation will help to elucidate disease mechanisms.

Since mAChR is a synaptic GPCR, its regulation may be mediated by other GPCRs, as this is a common mechanism of regulation in this superfamily of proteins (Hur & Kim 2002; Cordeaux & Hill 2002). For example, stimulation of the PLC-coupled P2Y2 receptor inhibits β2-adrenergic receptor-mediated cAMP production via G_i proteins (Suh et al. 2001). Furthermore, mAChRs and mGluRs can also participate in cross-talk signalling with other receptors. Illustrating this, activation of mAChR3 results in heterologous PKC-mediated phosphorylation of β2-adrenergic receptor, causing its desensitisation (Budd et al. 1999; Pera & Penn 2014). In addition, mGluR7 activation can inhibit β-adrenergic receptor-mediated adenylyl cyclase activation (Ferrero et al. 2016). Therefore, it is possible that inhibitory regulation of mAChRs may be exerted by mGluR5 activity. This concept

is supported by evidence showing mGluR5-mediated inhibition of mAChR1 in the perirhinal cortex (Jo et al. 2006). In addition, preliminary data from our lab supports the inhibition of mAChRs by group I mGluRs in stress- and Aβ-related pathologies (unpublished results) in the same brain region. However, whether this functional interplay occurs in the hippocampus is unknown.

Another key molecule in AD pathology that may be regulated by mGluR5 is glycogen synthase kinase 3β (GSK-3β). GSK-3β is a constitutively active kinase (Woodgett 1990; Eldar-Finkelman & Martinez 2011) that originally emerged as a critical mediator of neurodegeneration due to its ability to phosphorylate tau (Mandelkow et al. 1992; Wagner et al. 1996; Li & Paudel 2006; Sperber et al. 1995). Since the phosphorylation state of tau is known to regulate its aggregation (Pérez et al. 2002), it has been proposed that GSK-3β-induced tau phosphorylation is responsible for the formation of tau aggregates (Cho & Johnson 2004; Ando et al. 2016; Hernández et al. 2010; Busciglio et al. 1995). This is supported by studies showing that inhibition of GSK-3β results in reduced tau aggregation and degeneration *in vivo* (Noble et al. 2005; Pérez, Hernández, et al. 2003) and *in vitro* (Alvarez et al. 1999). This finding is relevant to AD given the known neurotoxic effect of tau aggregates (Iqbal et al. 1989; Lee et al. 1989).

The importance of GSK-3β for AD further relies in its involvement in synapse weakening pathways. Indeed, GSK-3β regulates synaptic function and long-term plasticity (Peineau et al. 2007) and therefore its dysregulation in pathology could lead to impairments in synaptic function. Consistent with this concept, the Aβ-inhibition of LTP requires the activity of GSK-3β (Jo et al. 2011), an effect that may be due to the activation of GSK-3β in an Aβ-dependent manner (Baki et al. 2004; Takashima et al. 1998). Although it is clear that GSK-3β critically regulates synapse dysfunction, the molecular determinants of its activation remain poorly understood. GPCR regulation of GSK-3β is a potential mechanism that may be relevant to AD pathology as mAChR activation results in inactivation of GSK-3β (Ma et al. 2013) and neuroprotective effects from Aβ neurotoxicity (Farías et al. 2004). In line with this, activation of mAChRs reduced glutamate-induced overactivation of GSK-3β (Ma et al. 2013). In addition, a peptide derived from prion protein (PrP), which mediates Aβ-effects through synaptic mGluR5 (Um et al. 2013; Haas et al. 2016; Hamilton et al. 2015), triggers neuronal cell death via increased GSK-3β activity (Pérez, Rojo, et al. 2003). The regulation of GSK-3β by

synaptic GPCRs coupled to PKC activation, such as mAChRs and mGluRs, is in agreement with studies showing that PKC activity can regulate GSK-3β function *in vitro* (Goode, N.; Hughes, K.; Woodgett, J. R.; Parkeri 1992; Shin et al. 2002; Tejeda-Muñoz et al. 2015; Christian et al. 2002). Therefore, synaptic GCPRs may regulate GSK-3β activity and this regulation may be altered under neurodegenerative circumstances, having synaptotoxic effects that impair synaptic plasticity.

In summary, deficits in synaptic function are a clear hallmark of AD pathology. Several studies point towards disease-triggered aberrant activation of mGluR5 as a mechanism by which such deficits may occur. However, these studies are based on the effects of blockade of mGluR5 in *in vitro* or *in vivo* Aβ models of AD and on the evidence showing that mGluR5 blockade rescues the Aβ-inhibition of LTP. Why mGluR5 blockade is beneficial in these conditions and how it contributes to Aβ-mediated effects on synaptic plasticity is not fully understood. In this thesis, it was hypothesised that activation of mGluR5 is sufficient to cause deficits in synaptic function, and particularly that it causes depression of baseline recordings and inhibition of LTP induction in acute hippocampal slices. Furthermore, as a possible mechanism by which mGluR5 activation may be detrimental for synaptic function, it was hypothesised that mGluR5 activation inhibits mAChR function, as it is unknown whether this functional interaction occurs in the hippocampus. Specifically, the hypotheses tested were that mGluR5 activation (1) reduces mAChR-mediated depression of fEPSPs elicited by CCh in acute hippocampal slices and (2) reduces mAChR-mediated calcium increase elicited by CCh in primary hippocampal neuronal cultures. Finally, another key molecule involved in AD pathology is GSK-3β and there are reasons to think that this kinase is regulated by GPCRs activity, such as mGluR5. Whether this is a potential mechanism in the hippocampus that may contribute to Aβ pathology is unknown. Accordingly, it was hypothesised that activation of mGluR5 results in the activation of GSK-3β in acute hippocampal slices. Altogether, results in this chapter will clarify whether mGluR5 activation is sufficient to cause similar effects on synaptic function as those seen in AD models (where mGluR5 is activated) and therefore will help to evaluate whether mGluR5 is a good therapeutic target in AD.

3.2 Results

3.2.1 mGluR5 activation does not affect baseline recording or LTP induction

Evidence suggests that an excessive activation of mGluR5 could serve as a possible molecular mechanism underlying Aβ-induced pathology, including disruption of synaptic plasticity (Kumar et al. 2015). It was first sought to determine whether agonist activation of mGluR5 can alter synaptic plasticity in non-disease (i.e. physiological) conditions that may lead to similar synaptic effects to those seen in the Aβ-induced pathological state. If mGluR5 activation is sufficient to cause Aβ-induced synaptic deficits, targeting mGluR5 itself rather than Aβ pathology may be more effective when designing therapeutic strategies to treat AD. To this purpose, field recordings in acute hippocampal slices were carried out to assess the effect of mGluR5 activation on baseline recording and LTP induction. Healthy slices are known to exhibit LTP and stable baseline recordings, therefore these were used as a measurement of synaptic function (Abrahamsson et al. 2016). In the first set of experiments, CHPG, which has been shown to act as a selective mGluR5 agonist (Doherty et al. 1997) was used. A concentration range of CHPG (EC_{50} = 7.3 μM) (Fazal et al. 2003) was tested consisting in 1, 10 and 50 μM applied for 20 minutes. These concentrations were chosen based on previously published work (Doherty et al. 1997; Neyman & Manahan-Vaughan 2008; Izumi & Zorumski 2012) to activate mGluR5 without eliciting LTD as this could affect LTP induction. Stimulation of Schaffer collaterals was used to evoke fEPSPs in the CA1 region that were recorded throughout the experiment. As explained in **Chapter 2** (**section 2.11.3**), fEPSPs were used as a measure of synaptic efficacy. No differences were found between the fEPSPs elicited in CHPG-treated slices and control slices, showing that CHPG did not have an effect on baseline recording at any of the concentrations used (1 μM: p-value = 0.474, 10 μM: p-value = 0.146, 50 μM: p-value = 0.0927, **Figure 3-1**).

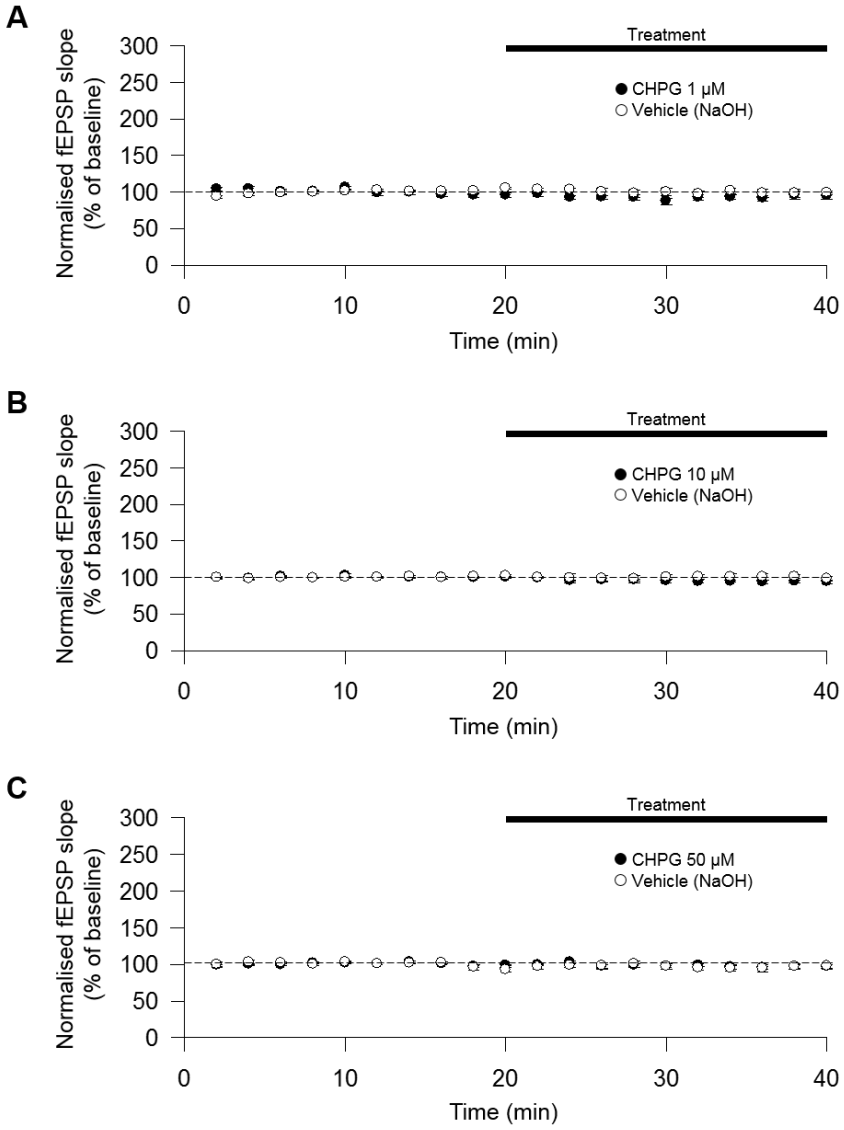


Figure 3-1. The selective mGluR5 agonist, CHPG, does not affect baseline recording in acute hippocampal slices. (A) Comparison between Schaffer inputs from control slices (N = 7) and CHPG 1 µM-perfused slices (N = 7) showed no effect of CHPG on basal recording of fEPSPs. **(B)** Comparison between Schaffer collateral inputs from control slices (N = 5) and CHPG 10 µM-perfused slices (N = 5) showed no effect of CHPG on basal recording of fEPSPs. **(C)** Comparison between Schaffer collateral inputs from control slices (N = 6) and CHPG 50 µM-perfused slices (N = 7) showed no effect of CHPG on basal recording of fEPSPs. In all graphs, data were normalised to first 20 minutes. Circles represent the mean and error bars the standard error of the mean (S.E.M.). Significance level (*$p < 0.05$) was determined by using an unpaired Student t-test between data points corresponding to minutes 36, 38, 40 between control and CHPG-perfused slices.

Given the role of mGluR5 on synaptic plasticity (Huber et al. 2000; Francesconi et al. 2004), it would be expected that agonist stimulation of mGluR5 impacts LTP induction. This hypothesis was tested by using CHPG, which was perfused at different concentrations (1, 10, 50 µM) for 20 minutes prior to delivery of HFS on

the Schaffer collateral input. CHPG was perfused for 10 minutes more after HFS delivery to ensure the treatment was effective during induction of LTP. No difference was found between fEPSP slopes recorded after HFS delivery from control slices and CHPG-perfused slices at any of the concentrations used (1 μM: p-value = 0.931, 10 μM: p-value = 0.811, 50 μM: p-value = 0.964, **Figure 3-2**). Therefore, CHPG perfusion did not affect LTP induction.

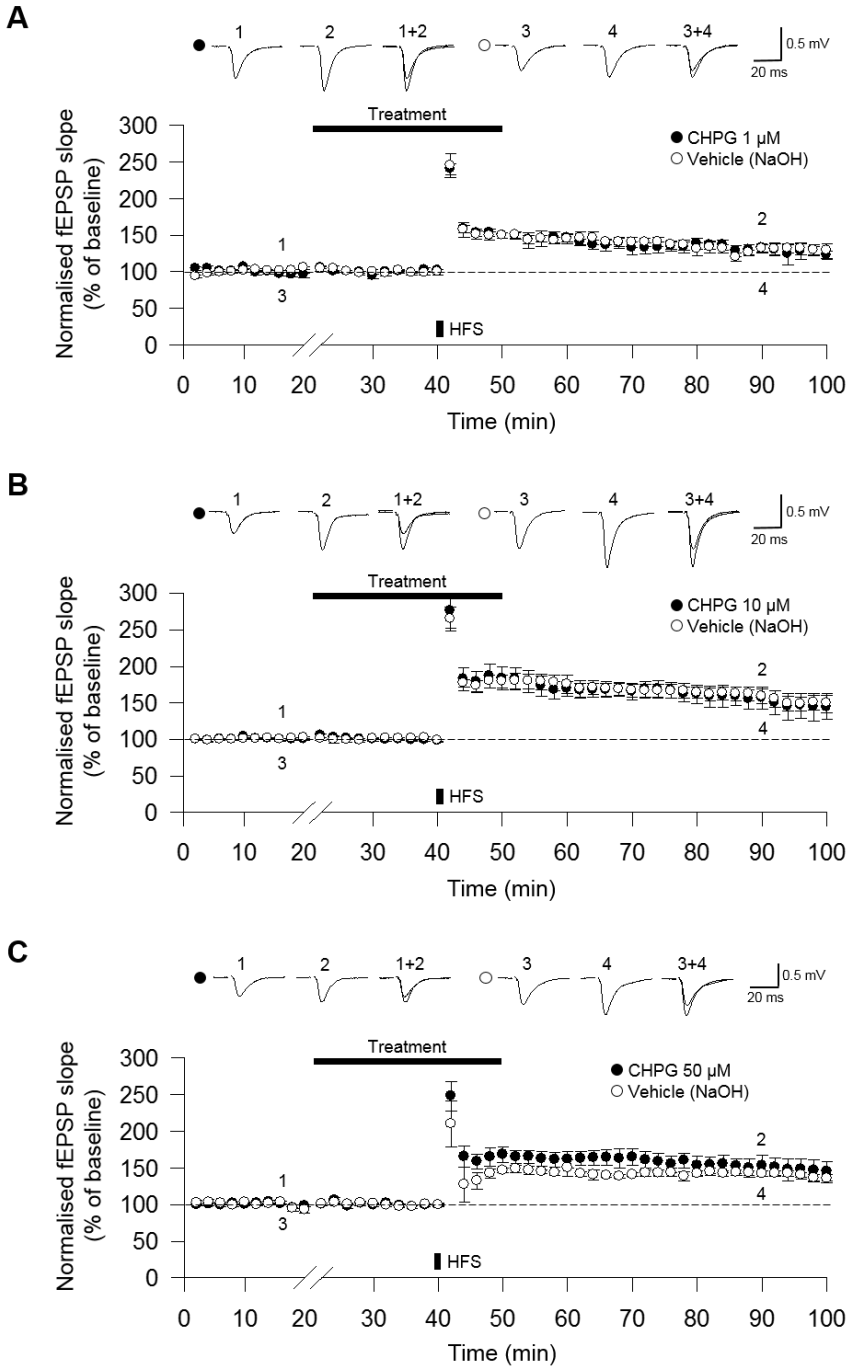


Figure 3-2. The selective mGluR5 agonist, CHPG, does not affect LTP induction in acute hippocampal slices. (A) LTP was induced in control slices (N = 7) and CHPG 1 µM-perfused slices (N = 7) **(B)** LTP was induced in control slices (N = 5) and CHPG 10 µM-perfused slices (N = 5). **(C)** LTP was induced in control slices (N = 5) and CHPG 50 µM-perfused slices (N = 6). In all graphs, data before the break were normalised to the first 20 minutes. Data after the break were normalised to the 20 minutes prior to HFS delivery. Top panel shows representative fEPSPs before and after HFS and the superposition of both. Circles represent the mean and error bars the standard error of the mean (S.E.M.). Significance level (*$p < 0.05$) was determined by using an unpaired Student t-test between data points corresponding to minutes 86, 88, 90, 92, 94 between control and CHPG-perfused slices.

To verify these data, mGluR5 was activated using a different protocol. A combination of DHPG (20 µM), a group I mGluR agonist that activates both mGluR5 and mGluR1 (Palmer et al. 1997; Wisniewski & Car 2002) together with JNJ (1 µM), which is an mGluR1 negative allosteric modulator (Lavreysen et al. 2004) was used. Concentrations of pharmacological agents were selected to provide 5x of the half maximal response in the case of JNJ (IC_{50} = 0.019 µM) (Fukunaga et al. 2007) and 40x of the half maximal response in the case of DHPG (EC_{50} = 0.5 µM) (Fazal et al. 2003). These concentrations were selected based on previously published work (Fitzjohn et al. 1999; Palmer et al. 1997) to avoid eliciting mGluR-dependent LTD as this would interfere with LTP induction. This treatment should serve as an alternative pharmacological approach to selectively activate mGluR5. In the second set of experiments, field recordings in acute hippocampal slices were carried out to assess the effects of DHPG+JNJ treatment on baseline recording and LTP induction. Similarly to the data obtained with CHPG, no differences were observed between control slices and DHPG+JNJ-perfused slices neither in baseline recording (p-value = 0.0754, **Figure 3-3A**) nor in LTP induction (p-value = 0.603, **Figure 3-3B**).

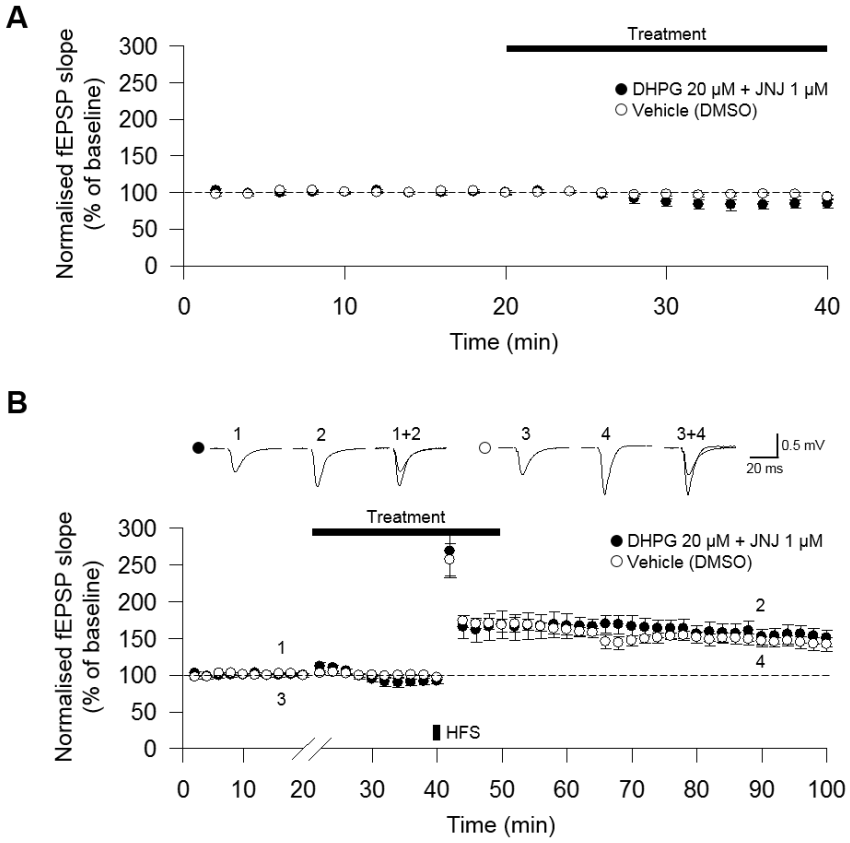


Figure 3-3. Selective activation of mGluR5 does not affect baseline recording or LTP induction in acute hippocampal slices. (A) Co-perfusion of slices (N = 6) with DHPG+JNJ (20 min) did not affect baseline recording as compared to control slices (N = 5). Data were normalised to first 20 minutes. **(B)** LTP was normally induced in control slices (N = 5) and DHPG+JNJ-perfused slices (N = 6). Data before the break were normalised to first 20 minutes. Data after the break were normalised to 20 minutes prior to HFS delivery. Top panel shows representative fEPSPs before and after HFS and the superposition of both. Circles represent the mean and error bars the standard error of the mean (S.E.M.). Significance level (*$p < 0.05$) was determined by using an unpaired Student t-test between data points corresponding to minutes 36, 38, 40 in A, or minutes 86, 88, 90, 92, 94 in B, between control and DHPG+JNJ-perfused slices.

Taken together, these data indicate that selective activation of mGluR5 does not affect synaptic transmission as assessed by baseline recording and LTP induction experiments.

3.2.2 Selective mGluR5 activation does not affect CCh-induced depression

Loss of cholinergic function is a hallmark of AD pathogenesis (Fisher 2012). The potential mGluR5-dependent inhibition of mAChR function could further lead to a deficiency in cholinergic signalling and contribute to AD pathology. This concept is supported by a study showing that aberrant expression of mGluR5 leads to the

inhibition of mAChR-mediated LTD in the perirhinal cortex (Jo et al. 2006) and by preliminary data in our lab (unpublished results).

First, it was sought to test whether a functional interaction between mGluR5 and mAChRs may exist in the hippocampus. To do this, the muscarinic agonist carbachol (CCh) in combination with CHPG were used. It is well established that perfusion of CCh during field recordings causes a long-lasting depression of the fEPSPs, an effect termed CCh-LTD (Kumar 2010; Massey et al. 2001; Volk et al. 2007; Kirkwood et al. 1999; Mccoy & Mcmahon 2007; McCutchen et al. 2006; Scheiderer et al. 2006). Although CCh activates both muscarinic and nicotinic receptors (Akay et al. 2009; Brenner & Stevens 2013), in the hippocampus this effect has been shown to be specifically mediated by mAChRs expressed postsynaptically and in particular by the mAChR1 subtype (Scheiderer et al. 2006; Kumar 2010; McCutchen et al. 2006). Therefore, CCh-induced depression was used as a readout of mAChR function. In agreement with these studies, all concentrations of CCh (50, 25, 10 µM) tested induced a depression of fEPSPs when perfused for 10 minutes during baseline recording experiments in acute hippocampal slices (**Figure 3-4**). It was next hypothesised that if mGluR5 could inhibit mAChR function, activation of mGluR5 with CHPG would have an effect on CCh-induced depression. However, no effect of CHPG on CCh-induced depression of fEPSPs was detected at any of the concentration tested (50 and 500 µM) as this depression was equally observed in CHPG+CCh-perfused slices and CCh alone-perfused slices (50 µM: p-value = 0.553, 25 µM: p-value = 0.35, 10 µM: p-value = 0.0756) (**Figure 3-4**).

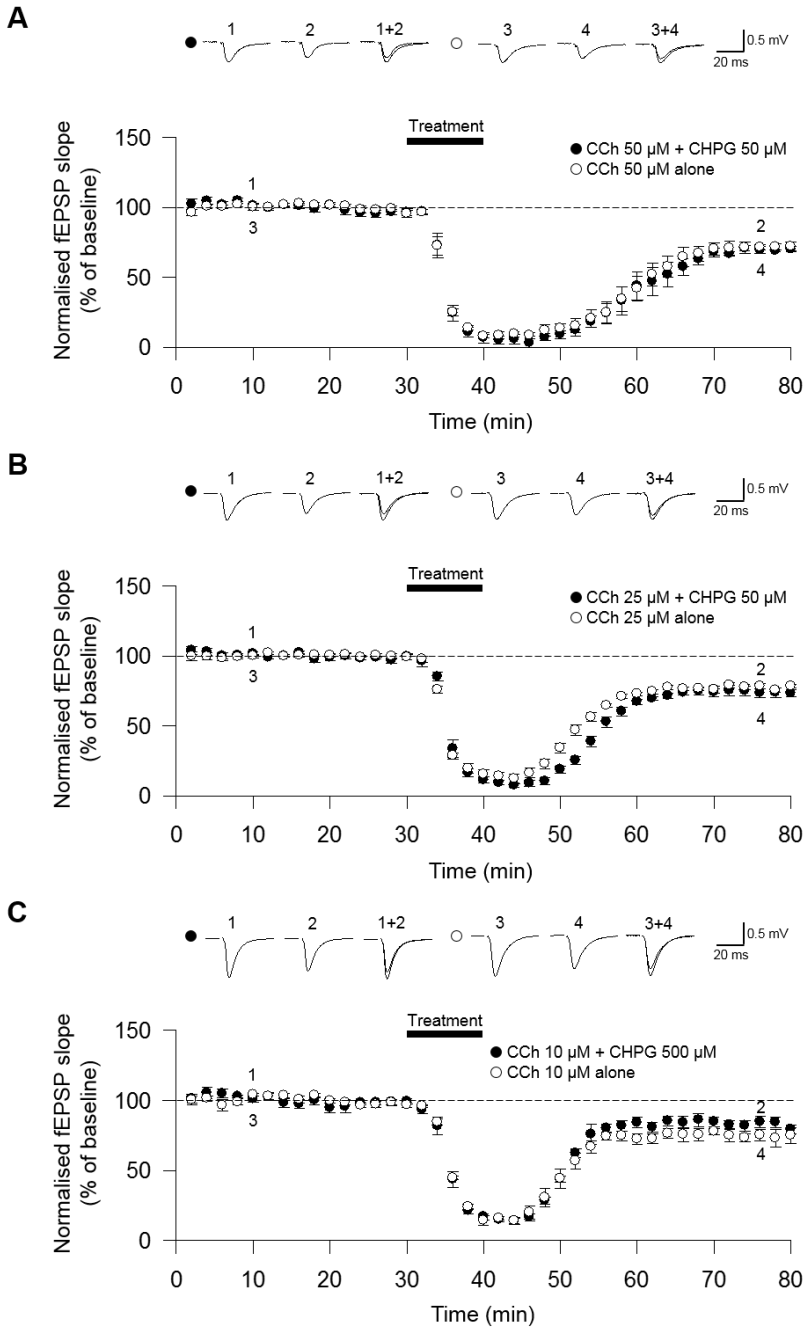


Figure 3-4. Perfusion of the selective mGluR5 agonist, CHPG, does not affect CCh-induced depression of fEPSPs in acute hippocampal slices. (A) Perfusion of CCh (50 µM, 10 min) resulted in the depression of fEPSPs slopes (N = 6) and this effect was not prevented by co-perfusion with CHPG (50 µM, 10 min) (N = 5). **(B)** Perfusion of CCh (25 µM, 10 min) resulted in the depression of fEPSPs (N = 5) and this effect was not prevented by co-perfusion with CHPG (50 µM, 10 min) (N = 6). **(C)** Perfusion of CCh (10 µM, 10 min) resulted in the depression of fEPSPs slopes (N = 5) and this effect was not prevented by co-perfusion with CHPG (500 µM, 10 min) (N = 6). Top panel shows representative fEPSPs before and after treatments and the superposition of both. Data normalised to first 30 minutes. Circles represent the mean and error bars the standard error of the mean (S.E.M.). Significance level (*p < 0.05) was determined by using an unpaired Student t-test between data points corresponding to minutes 72, 74, 76, 78, 80 between CCh alone and CCh+CHPG-perfused slices.

To exclude the possibility that activation of mGluR5 was required prior mAChRs activation to reduce CCh-induced depression, two alternative protocols were used. The first one involved perfusion of CHPG (50 µM) for 20 minutes prior CCh perfusion (10 µM for 10 minutes) and until the end of CCh application (a total of 30 minutes). Under these circumstances, CHPG did not affect CCh-induced depression (p-value = 0.965) (**Figure 3-5A**). The second protocol involved the incubation of acute hippocampal slices with an increased concentration of CHPG (100 µM) for 1-1.5 hours prior field recordings to ensure mGluR5 activation. Then CHPG was perfused from the start of recordings until the end of CCh application (total of 40 minutes). In these experiments, CHPG treatment had no effect on CCh-induced depression of fEPSPs (p-value = 0.164) (**Figure 3-5B**).

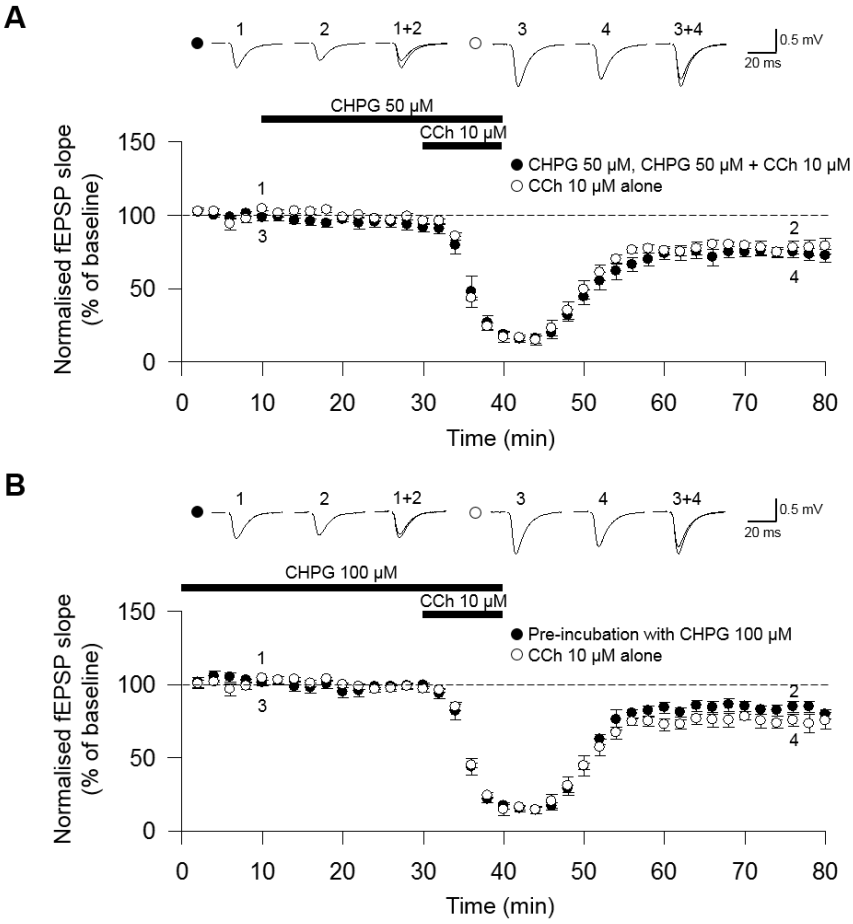


Figure 3-5. Pre-incubation or previous perfusion of acute hippocampal slices with the selective mGluR5 agonist, CHPG, do not affect CCh-induced depression. (A) Perfusion of CCh (10 µM, 10 min) resulted in the depression of fEPSPs slopes in acute hippocampal slices (N = 5). Perfusion of CHPG (50 µM) for 20 minutes prior perfusion of CCh (10 µM, 10 min) did not affect CCh-induced depression of fEPSPs (N = 5). **(B)** Pre-incubation of acute hippocampal slices with CHPG (100 µM, 1-1.5 h) and subsequent perfusion of CHPG during the recording for 30 minutes prior perfusion of CCh did not affect CCh-induced depression of fEPSPs (N = 7) compared to CCh alone-perfused slices (N = 7). Top panel shows representative fEPSPs before and after treatments and the superposition of both. Data normalised to first 30 minutes. Circles represent the mean and error bars the standard error of the mean (S.E.M.). Significance level (*$p < 0.05$) was determined by using an unpaired Student t-test between data points corresponding to minutes 72, 74, 76, 78, 80 between CCh alone and CCh+CHPG-perfused slices.

Taken together, these data indicate that activation of mGluR5 does not inhibit CCh-induced depression of fEPSPs in acute hippocampal slices, suggesting that mGluR5 does not inhibit mAChR function under these experimental conditions.

3.2.3 Effects of transient agonist activation of mGluRs on mAChR-mediated calcium increase

Electrophysiology data showed that agonist activation of mGluRs does not inhibit CCh-induced depression of fEPSPs in acute hippocampal slices. This suggests that mGluR5 may not inhibit mAChRs function under these experimental conditions. The activation of mAChRs by CCh involves the activation of the G-protein mediated pathway (Kelly et al. 1996) that results in an increase in intracellular levels of calcium (VanDeMark et al. 2006). Therefore, measuring the CCh-induced calcium increase would be a complementary approach to confirm the electrophysiology data.

According to this, the effects of agonist activation of mGluRs on mAChR-mediated calcium increase were assessed. Fluo4-AM is a cell-permeant calcium fluorescent dye that emits at 494/506 nm in its calcium-bound form and was used to load primary hippocampal neurons and detect changes in calcium concentration. First, it was established whether CCh treatment could produce an increase in intracellular calcium levels. As expected, acute activation of muscarinic receptors by CCh produced the characteristic increase in intracellular calcium (VanDeMark et al. 2009), that was significantly higher at 100 µM (160.02 ± 26.81, $p = 0.011$) and 200 µM (138.22 ± 15.02, $p = 0.026$) compared to 10 µM (91.59 ± 0.49) (Kruskal-Wallis one way ANOVA on ranks, $p = 0.007$, Dunn's method for all pairwise multiple comparison procedures, **Figure 3-6**).

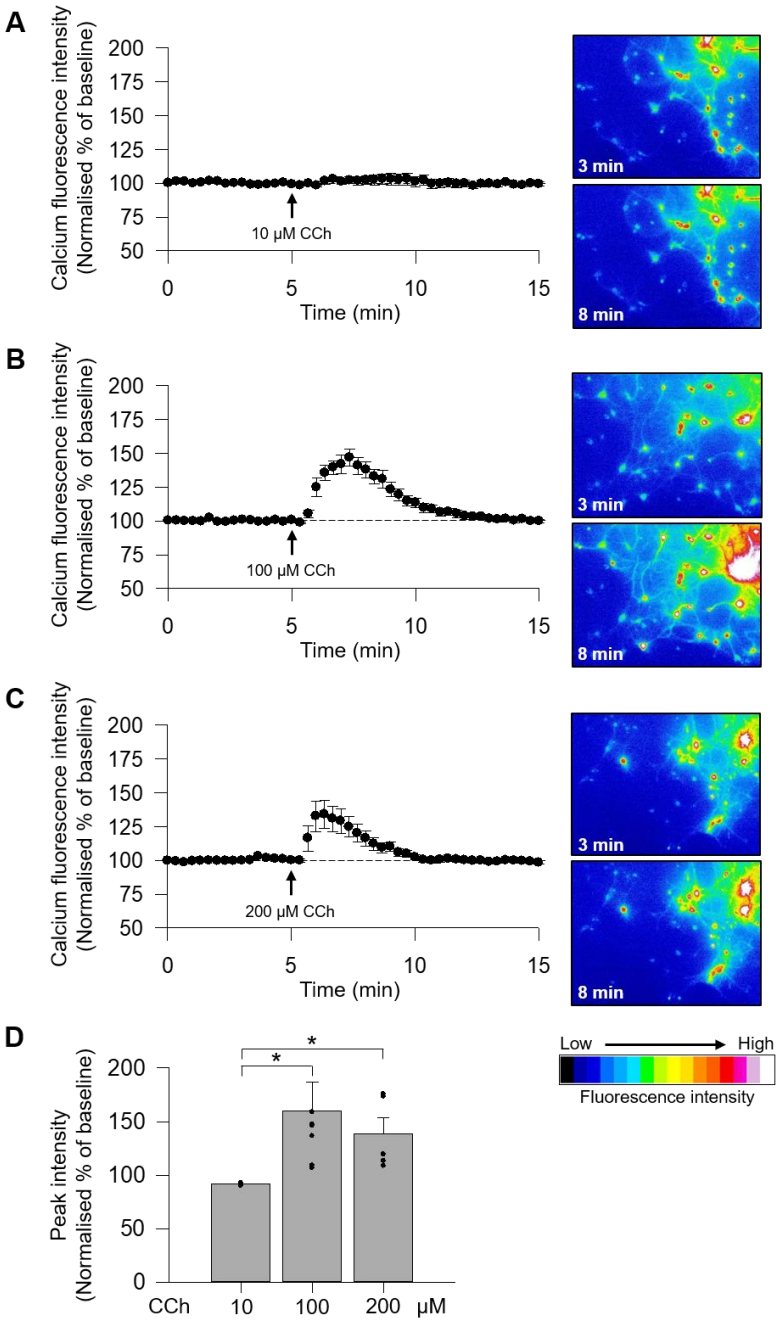


Figure 3-6. Effect of muscarinic agonist, CCh, on calcium concentration in primary hippocampal neurons. Acute application of muscarinic agonist, CCh, at the following concentrations: **(A)** 10 µM (N = 5 coverslips, 39 cells), **(B)** 100 µM (N = 7 coverslips, 54 cells), **(C)** 200 µM (N = 5 coverslips, 36 cells) caused an increase in the calcium concentration. Arrow indicates the addition of 5 mL of CCh prepared in HBS buffer. Right panel shows representative images of calcium imaging experiments. Differences in colour represent differences in Fluo-4AM fluorescence intensity (i.e. calcium concentration). Baseline and after-treatment images were taken at minute 3 and 8 of recordings, respectively. **(D)** Peak calcium fluorescence intensity for each condition. Circles represent averaged peak intensity of cells within one experiment (i.e. coverslip). Error bars represent the standard error of the mean (S.E.M.). Significance level (*$p < 0.05$) was determined by using a Kruskal-Wallis one-way ANOVA on ranks and a Dunn's test for multiple comparisons.

Next, the effect of agonist activation of mGluRs by DHPG on CCh-induced calcium increase was assessed. The perfusion of 20 µM DHPG throughout the experiment resulted in a reduction of the CCh-induced calcium increase ($p = 0.039$, **Figure 3-7A**). DHPG was perfused for 15 min before starting the recording to acquire a stable baseline as DHPG also caused an increase in intracellular calcium levels (**Figure 3-7B**). Neurons were depolarised after experiments with 90 mM KCl to confirm their viability (Gómez-Ramos et al. 2008) (**Figure 3-7C**). Taken together, these data show that activation of mAChRs by CCh results in an increase in intracellular calcium levels in primary hippocampal neurons and that this increase is reduced by activation of group I mGluRs with DHPG.

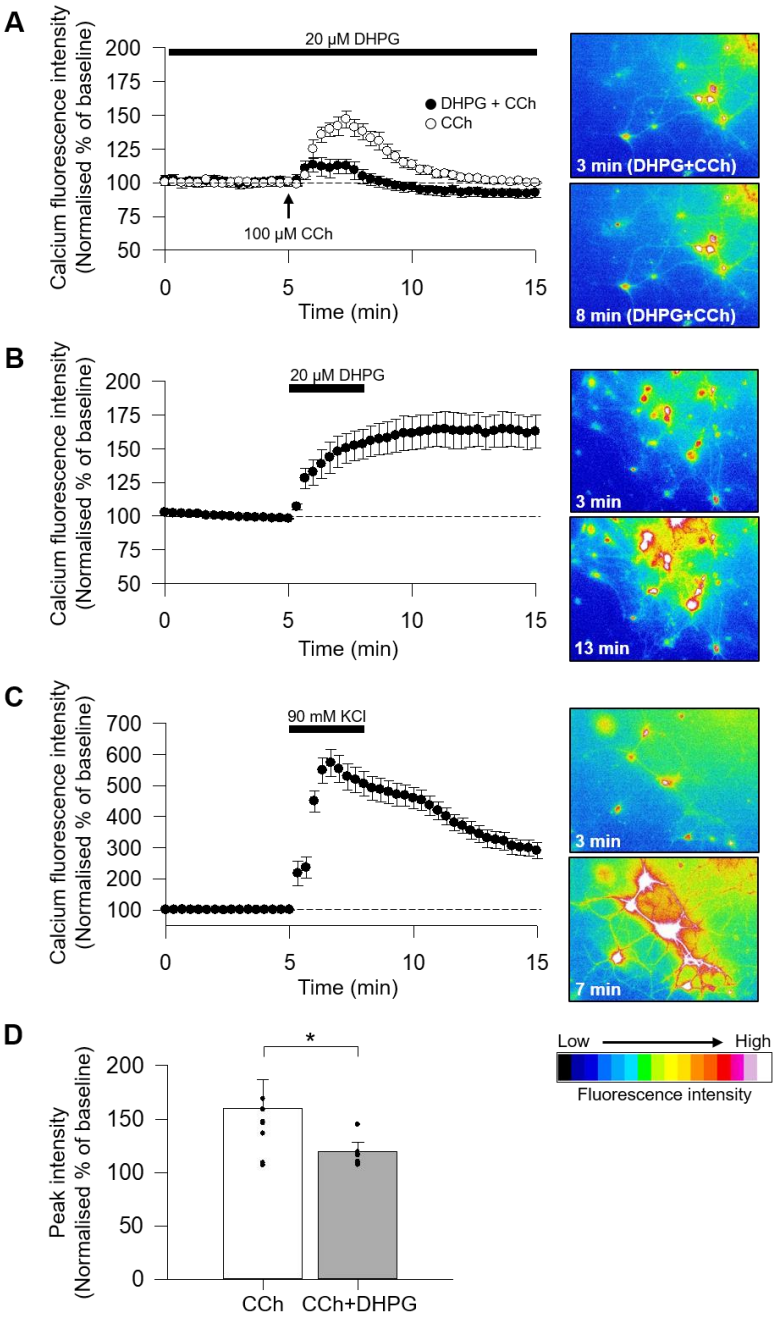


Figure 3-7. Perfusion of group I mGluRs agonist, DHPG, reduces CCh-induced calcium increase in primary hippocampal neurons. (A) Comparison between CCh 100 µM-perfused cells (N = 7, 54 cells) and CCh-perfused cells in combination with DHPG 20 µM (N = 6, 32 cells). DHPG was perfused for 10 min prior to starting the recording. **(B)** Application of DHPG 20 µM for 3 min (N = 4 coverslips, 17 cells). **(C)** Application of KCl 90 mM for 3 min (N = 4 coverslips, 30 cells). Right panel shows representative images of calcium imaging experiments. Differences in colour represent differences in Fluo-4AM fluorescence intensity (i.e. calcium concentration). Images were taken at the indicated times. **(D)** Peak calcium fluorescence intensity for each condition. Circles represent averaged peak intensity of cells within one experiment (i.e. coverslip). Error bars represent the standard error of the mean (S.E.M.). Significance level (*$p < 0.05$) was determined by using a Mann-Whitney rank sum test.

3.2.4 mGluR5 protein expression in acute hippocampal slices

Since electrophysiology data showed no effect of mGluR5 activation on baseline recordings or synaptic plasticity elicited in acute hippocampal slices (**Figure 3-1, Figure 3-2, Figure 3-3**), the possibility that this was due to relatively low expression of mGluR5 in this *in vitro* preparation was tested. To this purpose, various protein extraction conditions were tested and mGluR5 expression was analysed by western blot in acute hippocampal slices. Radioimmunoprecipitation assay (RIPA) buffer is a commonly used buffer for the lysis of cells in protein extraction protocols (Peach et al. 2012). However, the extraction of membrane-bound proteins such as mGluR5 may require harsher buffers that contain ionic detergents such as sodium dodecyl sulfate (SDS). According to this, two different buffers for cell lysis were used: RIPA buffer and sucrose/SDS buffer (see **Chapter 2**, **section 2.8.1**). It was also tested whether leaving samples with RIPA buffer for 1 hour improves the protein yield after tissue homogenization. Out of these three conditions, the sucrose/SDS buffer showed the best protein yield for mGluR5 (132 kDa) as revealed by western blot (**Figure 3-8**). These results indicate that mGluR5 protein is expressed in acute hippocampal slices. They do not demonstrate that mGluR5 is specifically expressed in CA1 pyramidal cells, where mGluR5 was expected to be activated in electrophysiology experiments. However, there is evidence supporting that mGluR5 is most abundant in the CA1 region of the hippocampus (Lujan et al. 1996). Considering this, findings here likely indicate that the absence of mGluR5 expression in the CA1 region of acute hippocampal slices used in this study does not explain the lack of effects of mGluR5 activation observed in field recording experiments.

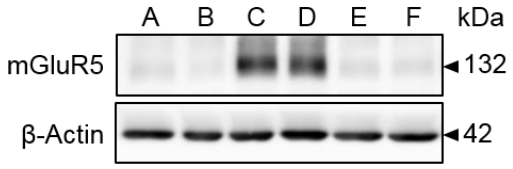


Figure 3-8. Optimization of mGluR5 total protein extraction from acute hippocampal slices. Conditions of protein extraction are as follows: lanes A and B: RIPA buffer, lanes C and D: Sucrose/SDS buffer, lanes E and F: RIPA buffer and samples left in rotating wheel at 4ºC for 1 h after tissue homogenization.

3.2.5 Agonist activation of mGluRs does not affect GSK3β activation

Work from our group and others have shown that GSK-3β is involved in synaptic weakening pathways underlying neurodegeneration (Eldar-Finkelman & Martinez

2011; Jo et al. 2011; Noble et al. 2005; Pérez, Hernández, et al. 2003). Regulation of GSK-3β may be controlled by synaptic GPCRs activation (Ma et al. 2013; Farías et al. 2004) likely via PKC activation (Goode, N.; Hughes, K.; Woodgett, J. R. ; Parkeri 1992; Shin et al. 2002; Tejeda-Muñoz et al. 2015; Christian et al. 2002). Importantly, this regulation may have an impact in neurodegenerative mechanisms (Farías et al. 2004). Because overactivation of mGluR5, a synaptic GPCR coupled to PKC activation, also contributes to synaptotoxicity (Kumar et al. 2015), the mGluR5-mediated regulation of GSK-3β is a potential mechanism of synaptic dysfunction. Accordingly, the hypothesis that activation of mGluR5 can result in the activation of GSK-3β in physiological conditions was tested. GSK-3β is a protein kinase that is activated upon dephosphorylation of the serine 9 (Ser9) residue (Sutherland et al. 1993; Stambolic & Woodgett 1994). Therefore, its activation can be determined by assessing protein levels of phosphorylated GSK-3β (P-GSK-3β) by western blot. First, acute hippocampal slices were incubated with 10 or 50 µM of DHPG for 30 minutes to activate group I mGluRs. None of these concentrations of DHPG influenced the levels of GSK-3β phosphorylation on Ser9 residue (control: 1.00 ± 0.14, DHPG 10 µM: 0.86 ± 0.15, DHPG 50 µM: 0.87 ± 0.12, $p = 0.718$, **Figure 3-9A**). Second, a time course of 50 µM DHPG was used to ensure that the absence of effect was consistent over time. Indeed, DHPG did not trigger activation of GSK-3β (Control: 1.00 ± 0.19, 10 min: 1.08 ± 0.19, 30 min: 0.99 ± 0.19, 2 h: 0.88 ± 0.07, 4 h: 0.83 ± 0.12, $p = 0.521$, **Figure 3-9B**). This data suggests that activation of group I mGluRs does not result in the activation of GSK-3β.

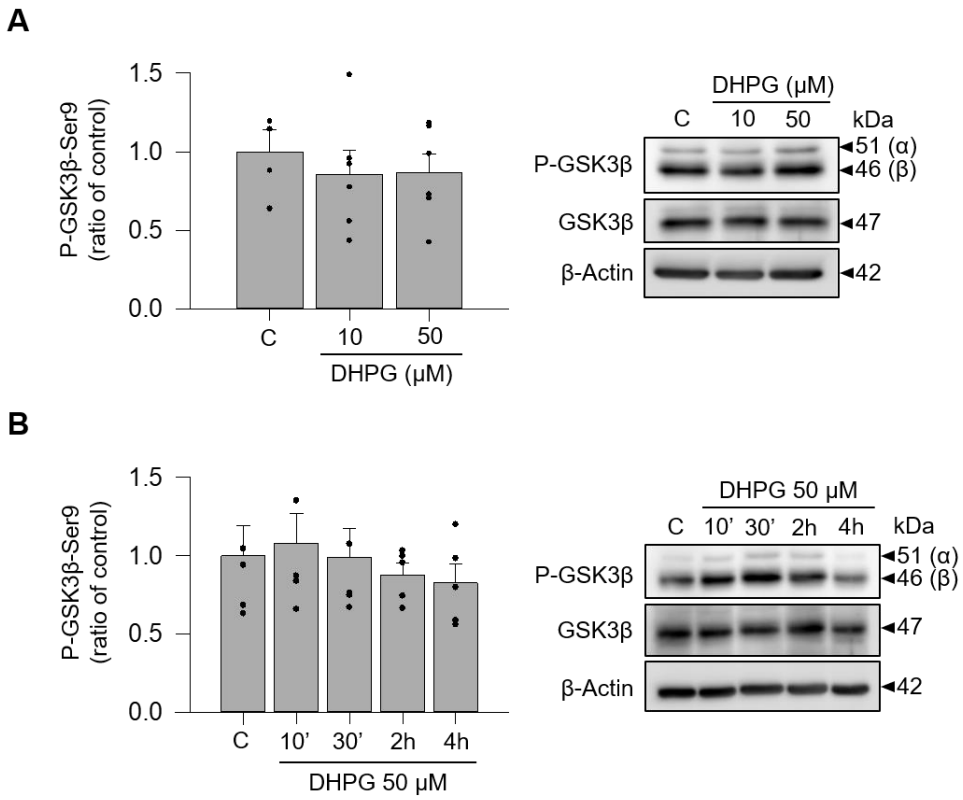


Figure 3-9. Incubation of acute hippocampal slices with DHPG does not affect GSK3β activation. (A) Concentration range of DHPG incubation (N = 6 for all groups). **(B)** Time course of DHPG 50 μM incubations (N = 5 for all groups). Insets show representative immunoblots. C: Control (aCSF only). Circles represent the mean and error bars the standard error of the mean (S.E.M.). Significance level (*$p < 0.05$) was determined by using a Kruskal-Wallis one-way ANOVA on ranks or one-way ANOVA.

3.3 Discussion

3.3.1 Activation of mGluR5: what makes it detrimental?

A main physiological role of mGluR5 is the regulation of synaptic transmission and plasticity (Lüscher & Huber 2010). Accordingly, excessive activation of mGluR5 may lead to synaptic impairments and this appears to be a plausible mechanism for Aβ-mediated effects on synaptic plasticity. However, whether mGluR5 activation is sufficient to cause Aβ-like impairments on synaptic function is unknown. It was therefore hypothesised that agonist activation of mGluR5 in non-disease conditions may lead to the same detrimental effects. To test this, field recordings in acute hippocampal slices perfused with the mGluR5 selective agonist CHPG were performed. At the concentration range used, CHPG did not cause any alterations on baseline recording (**Figure 3-1**) or LTP induction (**Figure 3-2**). To further corroborate this data, a different protocol was used to achieve mGluR5 activation. A treatment in which the combination of DHPG, a group I mGluR agonist that activates both mGluR5 and mGluR1, and the mGluR1 negative allosteric modulator mGluR1, JNJ, served as means of selectively activating mGluR5. This set of experiments yielded similar results to those obtained with CHPG, as no effect on fEPSPs was detectable on baseline recording or LTP experiments after co-perfusion of DHPG + JNJ (**Figure 3-3**). Together, this data suggests that, contrary to the initial hypothesis, agonist activation of mGluR5 does not lead to impairments in synaptic plasticity as per the measurements taken in this study.

One possibility to be considered is that the lack of effect on synaptic plasticity observed is due to a relatively low expression of mGluR5 in these experimental conditions. It has been shown that the amount mGluR5 protein reaches a peak during the first and second weeks of life followed by a decrease to the adult plateau level (Romano, van den Pol, et al. 1996). Rats used in this study were P24-P30, therefore the effect of agonist activation of mGluR5 with CHPG could be rather small as mGluR5 expression may not be high enough to see a clear functional effect. However, this is very unlikely because although the levels decrease after two weeks, this is in comparison to the levels at birth, which does not mean that expression after two weeks is non-existent or irrelevant for functional effects. Indeed, mGluR5 is highly localized in hippocampal dendritic fields of the stratum radiatum in the adult rat brain (Lujan et al. 1996). In agreement, western blot of acute hippocampal slices showed that mGluR5 was strongly expressed in this

preparation (**Figure 3-8**), eliminating its lack of expression as an explanation for the lack of effect of CHPG and DHPG on electrophysiological recordings.

Another consideration regarding the effects of activation of mGluR5 with agonists is that developmental differences in its expression are isoform-dependent. Three splice variants of mGluR5 mRNA have been described to date, namely mGlu5a, mGlu5b and mGlu5d, of which mGluR5a and mGluR5b are the best characterised (Joly et al. 1995; Minakami et al. 1995; Romano, Yang, et al. 1996; Malherbe et al. 2002). Studies have consistently shown that mGluR5a is predominantly expressed during postnatal stages while mGluR5b expression is higher in adult stages (Minakami et al. 1993; Romano, Yang, et al. 1996), with a similar dynamic distribution for protein and mRNA levels (Minakami et al. 1995; Joly et al. 1995; Romano, van den Pol, et al. 1996). Although agonist compounds that selectively activate one isoform are not available yet, the pharmacological characterisation of CHPG was done in mGluR5a-expressing cells (Doherty et al. 1997), the isoform of which expression is expected to decrease at the post-natal age studied here. Therefore, it could be possible that by using CHPG part of the activation within the population of mGluR5 is missing, specifically the mGluR5b variant, which precisely is thought to have a higher expression. However, this does not seem to be the case since no pharmacological differences have been described between mGluR5a and mGluR5b (Joly et al. 1995). In addition, data presented here shows that an alternative treatment to activate mGluR5 consisting in co-perfusion with DHPG, a group I mGluR orthosteric agonist that has no reported isoform preference, and JNJ, similarly showed no effects on field recordings (**Figure 3-3**). Therefore, this indicates that the lack of effect of mGluR5 activation is not related to preferential isoform activation by the compounds used in these experiments.

The above evidence shows that mGluR5 is abundantly expressed in the hippocampus, with higher levels than its group I counterpart, mGluR1 (Lujan et al. 1996). Given the role of the hippocampus in cognition, the expression of mGluR5 in this brain region has been linked to a role of the receptor in physiological mechanisms that mediate cognitive processes. Indeed, one of the main roles of mGluR5 in physiological conditions is the regulation of NMDARs, which are key mediators of higher cognitive functions (Riedel et al. 2003). Supporting this, pharmacological activation of group I mGluRs triggers the potentiation of NMDARs-induced depolarisations in CA1 (Fitzjohn et al. 1996) and this action

occurs via mGluR5 (Doherty et al. 1997; Mannaioni et al. 2001). Given the role of NMDARs in learning and memory (Riedel et al. 2003), the enhancement of NMDAR function by mGluR5 may be related to positive effects of mGluR5 activation on cognitive processes in physiological conditions (Homayoun et al. 2004; Stefani & Moghaddam 2010). It may be then the case that only when pathology is present that mGluR5 function is aberrantly engaged and consequently activation of the receptor is detrimental. In fact, much of the evidence supporting a role of mGluR5 in the pathology of AD is based on the neuroprotective effects of mGluR5 blockade, which are assessed under pathological conditions. For instance, Aβ peptides are commonly used to resemble AD-like brain pathology, and it is under these conditions when mGluR5 seems to participate in various Aβ-mediated pathological effects such as the facilitation and enhancement of LTD (Hu et al. 2014; Shankar et al. 2008) and impairment of LTP (Wang 2004; Rammes et al. 2011) as well as NMDAR-induced excitotoxicity (Movsesyan et al. 2001). In these studies, different forms of AD-related pathology are present in the experimental conditions where the effects of activation or inactivation of mGluR5 are assessed. A similar scenario is extended to *in vivo* evidence showing that genetic deletion (Hamilton et al. 2014) and pharmacological inhibition (Hamilton et al. 2016) of mGluR5 prevents memory deficits and Aβ pathology in an AD mice model. Therefore, this evidence suggests that mGluR5 activation mediates pathological effects in circumstances where the receptor is already in a diseased environment, such as the presence of toxic Aβ (Hu et al. 2014; Wang 2004) or overactivated NMDARs (Kingston et al. 1999; O'Leary et al. 2000). These molecules and/or pathways may interfere with mGluR5 signalling preventing its physiological actions with neurotoxic consequences. The results obtained in the present study support this concept by showing that activation of mGluR5 in non-pathological conditions does not affect synaptic plasticity, particularly LTP induction in the hippocampus (**Figure 3-2** and **Figure 3-3**). In agreement with the above evidence, this may be because the absence of AD-related pathological events renders mGluR5 activation as a harmless event for the neuron.

This knowledge is valuable to our understanding of molecular mechanisms operating in AD because it indicates that the involvement of mGluR5 is more complex than an on/off situation. Rather than this, a combination of pathology-triggered actions and/or molecules are simultaneously required for mGluR5 to become a detrimental insult for synaptic function. Importantly, this may mean that

although activation of mGluR5 alone is not sufficient to cause pathology in a healthy neuronal environment, inhibition of the receptor may be sufficient to restore normal synaptic function. This is in accordance with the central role of played by mGluR5 once the pathology is triggered. Therefore, identification of additional pathological events that accompany mGluR5 activation in AD will be crucial to successfully design therapeutic strategies aimed at this receptor.

3.3.2 mGluRs and mAChRs interplay

A synaptic hallmark of AD pathology is the reduction of cholinergic transmission (Whitehouse et al. 1982; Davies & Maloney 1976; Parent et al. 2013), which may be related to the observed reduction of mAChRs in the brains of AD patients (Shiozaki et al. 2001; Flynn et al. 1995). Supporting this, loss of mAChR1 results in increased Aβ-pathology in a mouse model of AD (Davis et al. 2010). This can potentially relate to the cognitive impairments that characterise AD, as blockade of mAChR1 results in cognitive impairment in mice (Anagnostaras et al. 2003) and agonism of mAChR1 rescues cognitive deficits in a mice model of AD (Caccamo et al. 2006). Therefore, identification of mechanisms by which activity or expression of mAChRs is reduced is of interest for the development of therapeutics aimed to restore mAChRs function in AD.

The impairment of mAChRs function mediated by mGluRs has been shown in the perirhinal cortex (Jo et al. 2006) and it is supported by preliminary data from our group (unpublished results). According to this, it was hypothesised that mGluR5 may also regulate mAChRs function in the hippocampus. To examine this, acute hippocampal slices were perfused with the muscarinic agonist CCh, which elicits a well-established depression of fEPSPs through the action of mAChRs (Kumar 2010; Massey et al. 2001; Volk et al. 2007; Kirkwood et al. 1999; Mccoy & Mcmahon 2007; McCoy et al. 2008; McCutchen et al. 2006; Scheiderer et al. 2006), in combination with the mGluR5 agonist CHPG. In these experiments, CHPG did not prevent the observed CCh-induced depression (**Figure 3-4**), even when slices were perfused or pre-incubated with CHPG for long periods of time (1-1.5 h) (**Figure 3-5**). The possibility that the concentration of CCh was too high that masked the effect of CHPG was considered. For this reason, a decreased concentration of CCh from 50 µM down to 10 µM was tested, as well as increasing concentrations of CHPG (up to 500 µM) (**Figure 3-4**). In none of these conditions, CHPG perfusion affected the CCh-induced depression by CHPG. Together these

data suggest that under these experimental conditions, activation of mGluR5 does not inhibit mAChR-mediated depression of fEPSPs in acute hippocampal slices.

These experiments may indicate that the interplay between mGluRs and mAChRs may not occur under these experimental circumstances or may not be of competitive nature. For instance, not competitive but cooperative signalling via mAChRs and group I mGluRs has been shown to increase the excitability of hippocampal CA1 pyramidal neurons when both receptors are synergistically activated (Park & Spruston 2012). In the experimental conditions of this study, however, a bigger magnitude CCh-induced effect when mAChRs and mGluR5 were co-activated was not observed. This suggests a lack of cooperative signalling for this particular functional aspect of mAChRs.

It is well established that both mAChRs (Levey et al. 1995) and mGluR5 (Lujan et al. 1996) are highly expressed in the CA1 region of the rat hippocampus, particularly in the dendritic fields. Nonetheless, the perisynaptic distribution of mGluR5 to the lateral margin of synapses and extrasynaptic locations have been well described (Lujan et al. 1996), whereas mAChRs are very likely enriched in the postsynaptic region of specific synapses (Levey 1996). Therefore, differential subcellular expression may potentially affect reciprocal interactions between receptors. This concept has been previously suggested as an explanation for how signalling pathways activated by GPCRs coupled to the same G proteins can exert different functional effects depending on their location (Moore et al. 2009; Ostrom & Insel 2004). For instance, activation of mGluRs and mAChRs results in the activation of extracellular signal-regulated kinase (ERK) in distinct compartments within the cell (Berkeley et al. 2001). In addition, there is evidence showing that GCPRs are specifically located in microdomains within the membrane, such as caveolae and lipid rafts (Insel et al. 2005). Illustrating this, G_q-coupled GPCRs and specifically group I mGluRs (Francesconi et al. 2009) and mAChRs (Gosens et al. 2007; Keshavarz et al. 2018), can localise to these domains where their signalling is enhanced (Calizo & Scarlata 2012). According to this, a plausible consideration regarding results presented here is that spatial separation of synaptic GPCRs located in different cellular compartments could result in the absence of competition for G-proteins and possibly other shared intracellular molecules.

In addition, GPCRs can signal through G-protein-independent pathways. For example, mGluRs can regulate brain function through non-GPCR mediators, such

as the non-receptor tyrosine kinases (Heuss & Gerber 2000; Ireland et al. 2004). In the experiments presented here, it could be possible that agonist activation of mGluR5 may trigger a G-protein independent pathway that may not interfere with the mAChR intracellular signalling that is involved in CCh-induced depression. One way to address whether there is an interaction between mGluRs and mAChRs at the level of intracellular signalling is to study changes in intracellular concentration of calcium induced by these receptors. It is very well-characterised that the activation by mAChRs by CCh results in an increase in intracellular calcium levels as a result of its release from intracellular stores (Gómez-Ramos et al. 2008; Haraguchi & Rodbell 1991; VanDeMark et al. 2009; Luo et al. 2001; Montiel et al. 2001; Mayerhofer et al. 1992) through the activation of the coupled G-protein (Caulfield & Birdsall 1998). Accordingly, calcium imaging in primary hippocampal neurons was carried out to test the hypothesis that mGluRs activation may impair calcium release triggered by mAChRs. Treatment of primary neurons with CCh induced the well-established increase in intracellular calcium levels, which was used as a readout of mAChRs function (**Figure 3-6**). Perfusion of group I mGluRs agonist DHPG caused a reduction in the CCh-mediated calcium increase (**Figure 3-7**), indicating that mGluRs may regulate mAChRs function under these experimental conditions.

The regulation of GPCRs function is exerted at many levels within the cell, including GPCR trafficking to the membrane, agonist binding, G-protein coupling, desensitisation and endocytosis of the receptor (Magalhaes et al. 2012). Out of these, receptor desensitisation can be seen as a central mechanism that is influenced by all other processes, resulting in a complex regulation of GCPRs signalling. GPCR desensitisation is triggered by various molecular mechanisms that have been classified as heterologous or homologous (Chuang et al. 1996). Homologous refers to agonist-dependent receptor desensitisation that generally involves the actions of GPCR kinases (GRKs) and arrestin proteins (Smith & Rajagopal 2016). These interactions generally result in the internalisation of the GPCR (Kelly et al. 2008). In opposition, heterologous desensitisation is agonist-independent, and it is triggered by the activation of other receptors rather than agonism of the GPCR that undergoes desensitisation (U. Klein et al. 2001; Cordeaux & Hill 2002). This latter mechanism of desensitisation could apply to data presented here, where mGluRs-activation results in the reduction of mAChRs-mediated calcium signalling.

One plausible mechanism of heterologous desensitisation is the competition for intracellular signalling complexes shared between group I mGluRs and mAChRs (Hur & Kim 2002; Hippe et al. 2013). Both receptors are coupled to $G_{q/11}$ and the generation of IP_3, which results in the release of calcium from intracellular stores (Huang & Thathiah 2015). Therefore, it is possible that application of DHPG activates this pathway through group I mGluRs that recruit part of the G-protein signalling complexes used by mAChRs. This mechanistic pathway would be further influenced by relative receptor abundance, since receptor density may be different for mGluRs and mAChRs. This can be a factor in determining the extent to which the more abundant GPCR sequesters G-proteins required by another GPCR present in lesser quantity (Willars et al. 1999; Bundey & Nahorski 2001). If mGluRs occupies a great part of the available GPCR signalling machinery, the later activation of mAChRs by CCh causes a smaller increase in intracellular calcium than if all downstream signalling molecules were available. Heterologous desensitisation of mAChRs can also occur as a result of uncoupling from $G_{q/11}$ proteins, a process reported to be triggered by α_{1B}-adrenoceptor activation (Bundey & Nahorski 2001). Since coupling of mAChRs to $G_{q/11}$ is required for the CCh-mediated effects on intracellular calcium (Gómez-Ramos et al. 2008; VanDeMark et al. 2009; Montiel et al. 2001; Lanzafame et al. 2003), mGluR-induced uncoupling of mAChRs would explain a reduction in the calcium response triggered by CCh.

Alternatively, it could also be the case that previous sustained activation of mGluRs results in the depletion of intracellular calcium stores so that less calcium is available to be released when mAChRs are later activated by CCh. The relevance of this effect would depend on two factors. First, whether there is a common intracellular calcium store shared between the receptors. This seems plausible since a cell contains many stores of calcium rather than one large reservoir and that movement of calcium in discrete stores is GPCR-driven by different agonists, allowing cross-talk between receptors (Blaustein & Golovina 2001). Second, the temporal dynamics of the intracellular store to replace the released calcium before further release is triggered (Werry et al. 2003). Interestingly, activation of group I mGluRs may result in the refilling of intracellular calcium stores through a L-type calcium channel-mediated mechanism (Fagni et al. 2000). Therefore, in the present experiments calcium stores could be replenished by the time mAChRs are activated, although this would depend on the timing of these two processes. In the

case that intracellular calcium stores are replenished shortly after calcium is released due to mGluR activation, calcium would be available for further mAChR-induced release. If this release is decreased by DHPG, as shown by data presented here, it may mean that mGluRs activation is causing the reduction of mAChRs available to mediate CCh-induced calcium increase. The loss of functional mAChR in the plasmatic membrane could occur through an increased desensitisation of the receptor induced by mGluRs. Interestingly, it has been shown that mAChRs activation can result in the heterologous internalisation of mGluRs in a CCh dependent manner (Mundell et al. 2002; Mundell et al. 2004). Therefore, a similar mechanism but in the opposite direction could be triggered by prior activation of mGluRs, resulting in mAChRs internalisation. This would result in a reduced number of mAChRs expressed in the plasmatic membrane that are available to bind CCh.

Overall, calcium imaging data supports an interplay between mGluRs and mAChRs that was not observed at the electrophysiological level. However, these results may not necessarily contradict each other. They may simply reflect differences in the mechanisms that produce the outcome used to asses mAChRs function, i.e. fEPSPs or calcium release. In field recording experiments, the generation of fEPSPs requires extracellular calcium (Error! Reference source not found.) in addition to intracellular calcium as well as other mechanisms that alter membrane excitability (Dong & Graziane 2016). In calcium imaging experiments, mobilisation of intracellular calcium accounted for the effect of mAChRs activation. Therefore, the interplay between mGluRs and AChRs could still happen in acute hippocampal slices although a functional effect was not seen by the specific measure of fEPSPs.

3.3.3 mGluRs and GSK3β

Finally, another possible mechanism by which mGluRs contributes to AD pathological processes is the activation of GSK-3β, a kinase involved in synapse-weakening pathways leading to neurodegeneration (Jo et al. 2006; Baki et al. 2004; Takashima et al. 1998; Hernández et al. 2010; Busciglio et al. 1995; Pérez, Rojo, et al. 2003). To test the hypothesis that mGluRs can regulate GSK-3β activity, acute hippocampal slices were incubated with different concentrations of DHPG to activate group I mGluRs. This set of experiments showed that regardless the concentration or incubation time, DHPG had no effect on GSK-3β activation as

assessed by the phosphorylation state of the protein in physiological conditions (**Figure 3-9**).

As outlined before, the pathological relevance of mGluR5 activation may only emerge in the situation where pathology is present and engages aberrant signalling of the receptor. Therefore, mGluR5-induced activation of GSK-3β may only occur in neurodegenerative conditions but not under physiological conditions, such as those of this study. This concept is supported by studies showing that pharmacological inhibition of mGluR5 results in increased inhibitory phosphorylation of GSK-3β at Ser9 in a mice model of FXS but not in wild-type mice (Min et al. 2009; Yuskaitis et al. 2010). This indicates that in conditions where mGluR5 is aberrantly activated, such as in FXS (Bear et al. 2004; Ronesi et al. 2012), the receptor can trigger the activation of GSK-3β. A possible link between GSK-3β and mGluR5 is the activation of NMDARs. It is known that NMDARs can regulate GSK-3β function, as blocking NMDARs results in increased inhibitory phosphorylation of GSK-3β (De Sarno et al. 2006). In addition, the regulation of NMDARs by mGluRs is well-established (Awad et al. 2000; Fitzjohn et al. 1996; O'Connor et al. 1994; Attucci et al. 2001), so excessive activation of mGluR5 could lead to NMDAR-induced activation of GSK-3β in disease conditions. Therefore, although data presented here does not support that activation of mGluR5 induces activation of GSK-3β in non-disease conditions, the possibility that this is still a plausible mechanism in AD pathology cannot be excluded.

3.3.4 Summary

In summary, according to data presented in this chapter, activation of mGluRs in physiological conditions does not seem to have detrimental effects on synaptic plasticity mechanisms such as LTP. Neither it affects the activation of synapse-weakening pathways mediated by GSK-3β. This does not exclude that in AD-like conditions, when the receptor is aberrantly activated and non-physiological mechanisms may be triggered, the activation of mGluR5 is harmful for the neuronal environment. Evidence provided here supports that group I mGluRs may inhibit mAChR function in physiological conditions. The relevance of this interplay for AD pathological mechanisms can be speculated based on deficits in cholinergic transmission and pathological activation of mGluR5 described in disease conditions. Therefore, the mGluR-mediated inhibition of mAChRs function would be particularly important if the expression or function of mGluRs is affected in AD.

For this reason, the next chapter is aimed to explore the changes in synaptic GPCRs and related proteins in the brains of AD patients at different stages of pathology.

Chapter 4 Synaptic protein expression in Alzheimer's disease brains

4.1 Introduction

4.2 Results

4.2.1 Synaptic protein expression in post-mortem human brain from AD patients

4.2.2 Co-immunoprecipitation between mGluR5 and Homer 1b/c

4.2.3 Gene expression of synaptic proteins in post-mortem human brain from AD patients

4.2.4 Synaptic protein expression in post-mortem human brain from P-AD patients

4.3 Discussion

4.1 Introduction

Alzheimer's disease is a neurodegenerative disorder characterised by a long pathological progression that starts many years before the clinical diagnosis of dementia (Sperling et al. 2011; Price & Morris 1999). This pathology includes the accumulation of the two AD-defining molecules, Aβ and hyperphosphorylated tau, in the form of amyloid plaques and NFTs in the brains of AD patients, respectively (Perl 2010). The anatomical distribution of these lesions is characterised by following a specific pattern and selectively affecting some neuronal populations (Braak & Braak 1991; Braak et al. 1993; Braak et al. 2006; Thal et al. 2000; Thal et al. 2002). Importantly, once triggered, the neuropathological process progresses relentlessly for decades until cognitive dysfunction is observable in the form of clinical symptoms (Vickers et al. 2016). Since gradual brain deposition of pathological Aβ and hyperphosphorylated tau aggregates occurs in a predictable sequence, its use as a diagnostic tool to determine the stage of pathology has been proposed (Sperling et al. 2011). Although the therapeutic importance of detecting AD at early stages is undeniable (Korolev 2014), even more relevant is to identify the molecular changes occurring at different stages, so therapeutic interventions can be tailored to these specific phases.

The fact that AD pathology gradually takes over brain structures has led to think that neuronal dysfunction in affected regions also occurs progressively, hence the definition of AD as a "progressive" neurodegenerative disorder (Vickers et al. 2016). Therefore, the need to understand how factors underlaying neurodegeneration evolve during the course of AD has become essential. It is widely believed that neurodegeneration results in deterioration of synaptic function that eventually leads to synaptic loss, and this has been linked to the severe impairments in cognitive abilities in AD (Masliah & Terry 1993; Masliah et al. 1989; Scheff & Price 1998; Scheff et al. 2006; Scheff et al. 2001; Terry et al. 1991; DeKosky et al. 1996; DeKosky & Scheff 1990). Support for this comes from studies showing a correlation between synaptic loss in AD brains and cognitive decline (De Wilde et al. 2016; Terry et al. 1991; DeKosky et al. 1996; DeKosky & Scheff 1990). In fact, synapse loss has become a hallmark not only for AD but for multiple types of dementia (Scheff et al. 2014). Notably, the integrity of synapses relies to a great extent on the correct functioning of neurochemical communication (Mckinney 2010), which depends on different neurotransmitter systems across brain regions (Kandimalla & Reddy 2017). In the hippocampus, one of the first brain regions

affected in AD (Braak & Braak 1991; Braak et al. 2006), glutamatergic transmission plays a key role in memory processing (Squire & Zola-morgan 1991; Bird & Burgess 2008) and glutamate receptors are critically involved in the maintenance of dendritic spines (McKinney et al. 1999; Ultanir et al. 2007; Mateos et al. 2007). Therefore, understanding the vulnerability of glutamate receptors in AD may hold important insights regarding disease mechanisms and identification of new therapeutic targets.

As outlined in **Chapter 1**, glutamatergic transmission is mainly mediated by two types of receptors, ionotropic and metabotropic. The involvement of these receptors in the pathology of AD is widely accepted and has been the object of intense research, but how they operate in pathologies is not fully understood yet. The involvement of NMDARs in AD has been linked, among other things, to their ability to mediate calcium excitotoxicity. It is well-established that chronic activation of NMDAR leads to a prolonged calcium influx into the postsynaptic neuron (Choi 1987; Greenamyre et al. 1988; Dong et al. 2009). Importantly, calcium excitotoxicity has been proposed as a mechanism for Aβ-mediated neuronal death (Demuro et al. 2005; Mattson et al. 1992; Alberdi et al. 2010; Arias et al. 1995; Le et al. 1995). Indeed, NMDAR-dependent calcium influx causes a rise in intracellular calcium levels that activates various intracellular cascades resulting in cell death (Alberdi et al. 2010; Kelly & Ferreira 2006; Harkany et al. 2000). This aberrant activation of NMDAR can be triggered by glutamate excessively accumulated at the synapse (Parpura-Gill et al. 1997; Fernández-Tomé et al. 2004). Excessive accumulation of glutamate in turn can occur by Aβ-induced decrease in glutamate clearance (Li et al. 2009; Parpura-Gill et al. 1997; Fernández-Tomé et al. 2004). This process involves Aβ-mediated impairment of the function or expression of glutamate transporters located in neurons and glia, resulting in increased extracellular levels of glutamate (Li et al. 2009; Fernández-Tomé et al. 2004). In addition, Aβ can induce synaptic glutamate release in a potassium- and calcium-dependent manner (Kabogo et al. 2010; Arias et al. 1995; Abramov et al. 2009). Crucially, these findings are supported by human studies showing reductions in glutamate transporters in AD brains (Lauderback et al. 2001; Li et al. 1997; Jacob et al. 2007; Scott et al. 2011; Kashani et al. 2008; Kirvell et al. 2006). In addition to elevated glutamate levels, NMDAR can also be activated in pathological conditions in a more direct way. Illustrating this, Aβ can interact with NMDAR (De Felice et al. 2007; Texidó et al. 2011; Alberdi et al. 2010) to induce neurotoxic effects that are

detected preferentially on glutamatergic neurons (Deshpande et al. 2009; Sokolow et al. 2012). Further supporting a central role for NMDARs in AD, one of the FDA-approved treatments for AD is the NMDAR blocker memantine (Alzheimer's Association 2018). This evidence therefore indicates that NMDARs are dysregulated in the presence of AD-like pathology.

Synaptic dysfunction is associated with impairments in cognitive processes such as memory. Therefore, pathological alteration of synaptic plasticity mediators such as AMPARs (Shankar et al. 2008) is likely to have an impact on cognition. In support of this, Aβ-mediated enhancement of LTD involves the removal of AMPARs from postsynaptic membranes in a NMDAR-dependent manner (Hsieh et al. 2006; Shankar et al. 2007). This may be related to the Aβ-mediated impairment of AMPAR trafficking (Gu et al. 2009) and may be relevant for synaptic function since blocking AMPAR endocytosis prevents the Aβ-mediated loss of dendritic spines (Hsieh et al. 2006). In addition to amyloid pathology, tau pathology also contributes to the disruption of AMPAR function. For instance, there is evidence for a reduction of AMPAR expressed in the postsynaptic membrane in a tau model of AD (Hoover et al. 2010) which may be related to the ability of tau to interfere with AMPAR trafficking (Jurado et al. 2018). Furthermore, tau can also disrupt AMPAR-dependent synaptic plasticity (Fá et al. 2016; D'Amelio et al. 2011) in AD models. Overall, this evidence shows that dysregulation of ionotropic glutamate receptors, AMPARs and NMDARs, plays a key role in pathological mechanisms associated with AD.

mGluRs also contribute to glutamatergic transmission and memory processing. As explained in **Chapter 3**, compelling evidence supports that mGluRs and particularly the mGluR5 subtype are important mediators of AD-related pathological effects (Kumar & Singh 2015). Part of this evidence is based on the positive effects of mGluR5 activation on the Aβ-precursor APP secretion (Lee et al. 1995; Sokol et al. 2011). In addition, mGluR5 has been shown to mediate the detrimental effects of Aβ on synaptic function, including the blockade of LTP (Walsh et al. 2002; Shankar et al. 2008; Jo et al. 2011; Rammes et al. 2011) and the enhancement of LTD (Hu et al. 2014; Hsieh et al. 2006; Li et al. 2009; Shankar et al. 2008). Importantly, this may have behavioural effects as suppression of mGluR5 activity rescued deficits in learning, memory and spine density triggered by Aβ (Um et al. 2013; Hamilton et al. 2016; Hamilton et al. 2014). As in the case

of NMDARs, mGluR5 can also behave as a receptor of Aβ in the synaptic membrane which may provide a mechanistic explanation for how Aβ triggers aberrant activation of mGluR5 (Um et al. 2013; Haas et al. 2016; Hamilton et al. 2015; Renner et al. 2010). These studies highlight the importance of glutamate receptors in mediating AD-associated neuropathology such as Aβ-toxicity.

Most of the evidence presented above relies on animal models of disease, which have several limitations and do not reflect the true complexity of the disease (Franco & Cedazo-Minguez 2014). Although these models allow for useful manipulations to study molecular disease mechanisms, they are imperfect in mimicking disease pathology in human patients (King 2018). For instance, synapse loss and dysfunction, which are now believed to be a core triggering factor for early memory impairment, are not often observed in transgenic models of AD (Hu et al. 2003; King & Arendash 2002). This may be a main difficulty when translating pre-clinical findings into successful treatments for patients. Furthermore, animal models are usually generated from genetic mutations and may not reflect the complex multifactorial origin of the most common form of AD, the sporadic variant. In addition, animal models tend to exhibit just a few aspects of AD pathology, hampering a comprehensive assessment of how receptors are affected by pathological mechanisms and also the effects of drugs in the symptomatology/progression of the disease (Franco & Cedazo-Minguez 2014). Moreover, disease progression in AD animal models occurs in a very different time window than in AD patients (Dam & De Deyn 2011; Franco & Cedazo-Minguez 2014). These hurdles may explain why pharmacological agents designed according to beneficial changes (of molecular or cognitive nature) in animal models may behave differently when tested in humans. In this regard, a main consideration is that human brains have been subjected to a much more heterogeneous pathology for longer periods of time.

Therefore, AD remains being a disorder that affects humans only and consequently research towards the study of AD patient's brains is gaining importance (Perl 2010). For example, *in vivo* receptor binding studies with positron emission tomography (PET) probes in human brains provide information about the specificity and dose requirements of new drugs during clinical trials (Ametamey et al. 2007; Terbeck et al. 2015; Pillai & Tipre 2016). They also help to study the mechanism of action of drugs and receptor density in the diseased brain (DeLorenzo et al.

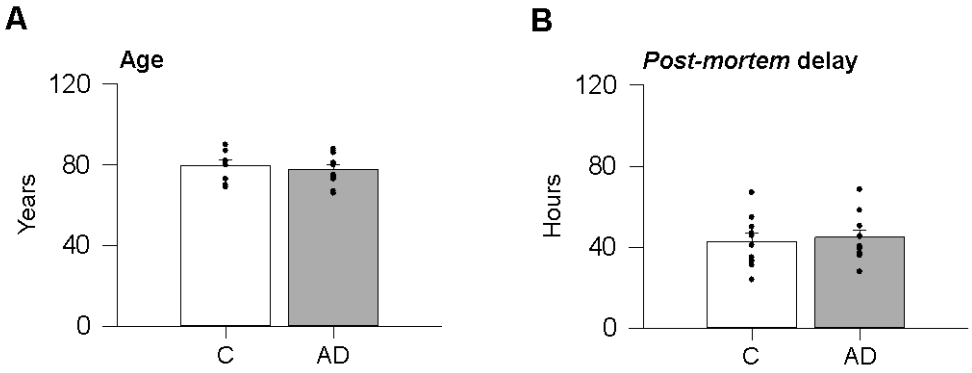
2011; Treyer et al. 2008; Akkus et al. 2014; Deschwanden et al. 2011; Albasanz et al. 2005). In addition, protein and gene expression studies in *post-mortem* tissue can provide direct information of the state of receptors at different stages of the disease (Albasanz et al., 2005; Gomez-Nicola & Boche, 2015; Gylys, 2004; Haas et al., 2016a; Scarr, McLean, & Dean, 2016; Sokolow et al., 2012; Tiwari et al., 2016).

As previously outlined, Aβ and tau pathologies and associated neuronal dysfunction are thought to gradually develop in AD. Therefore, it can be hypothesised that glutamate receptors with a demonstrated involvement in these pathologies, at least in AD models, may also be progressively affected as disease develops in the human brain. Accordingly, it is reasonable to hypothesise that glutamate receptor expression may change over the course of AD as a consequence of differential regulation by pathological mechanisms. Although synapses and thus synaptic receptors may be particularly vulnerable at early stages of AD (Selkoe 2002; Revett et al. 2013), it is unknown whether this represents an overall loss of total protein levels and whether this loss in maintained throughout disease progression. This is important because successful pharmacological manipulation of synaptic glutamate receptors made in animal models of AD may not necessarily be an efficient approach in humans if overall receptor proteins are lost in AD brains. Moreover, since data from **Chapter 3** has shown that mGluRs can inhibit mAChRs function, the relevance of this functional interplay in AD may be subject to changes in protein expression. The hypotheses being that (1) upregulation of mGluR5 protein levels and (2) downregulation of mAChRs protein levels occur in the brains of patients with severe AD. The aim of this chapter is to test these hypotheses by analysing protein levels of glutamate and mAChRs receptors in *post-mortem* brain samples from human AD patients at two stages of AD pathology. These stages are defined as possible/probable AD (P-AD cohort) and severe AD (AD cohort). Findings from this study will help to understand how receptor expression may change over disease progression and why some pharmacological manipulations may be more effective in certain phases of AD. Hopefully, it will also lead to more representative models of AD and therefore better therapeutics designs.

4.2 Results

4.2.1 Synaptic protein expression in post-mortem human brain from AD patients

Samples from AD patients (Braak stages V-VI) and control subjects were received from the South West Dementia Brain Bank. There were no significant differences in age (C: 79.80 ± 2.52, AD: 77.70 ± 2.52, p-value = 0.563) and *post-mortem* delay (C: 1.04 ± 0.10, AD: 0.75 ± 0.09, p-value = 0.715) between control and AD groups (**Figure 4-1**), indicating that this will likely not account for any differences detected in the analysis. Protein levels of ionotropic glutamate receptor subunits were first analysed. To assess protein levels of AMPAR, an antibody against the GluA2 subunit of these receptors was used. Western blot analysis showed a significant decrease in GluA2 protein levels in AD samples compared to control samples (C: 79.80 ± 2.52, AD: 77.70 ± 2.52, p-value = 0.043, **Figure 4-2A**). Protein levels of NMDAR were assessed by using an antibody against the NR1 subunit, which is an obligated subunit to form a functional NMDAR heteromeric complex. Western blot analysis with this antibody showed a significant decrease in NR1 protein levels in AD patients compared to control subjects (C: 0.97 ± 0.11, AD: 0.54 ± 0.07, p-value = 0.005, **Figure 4-2B**). Overall, these findings suggest a loss of GluA2-containing AMPARs and NR1-containing NMDARs in AD.



C

Group	Age (mean)	Age (SEM)	PM delay (mean)	PM delay (SEM)	Females	Males	Total
C	79.8	2.5	42.9	4.0	5	5	10
AD	77.7	2.5	44.9	3.7	5	5	10

Figure 4-1. Age and *post-mortem* delay distribution of *post-mortem* AD samples. (A) No significant differences in age were found between the control (C) group (N = 10) and the Alzheimer's disease (AD) group (N = 10) **(B)** No significant differences in *post-mortem* delay were found between the C group (N = 10) and the AD group (N = 10). Circles represent the value for each subject and error bars represent the standard error of the

mean (S.E.M.). Significance level (*p < 0.05) was determined by using an unpaired Student t-test. **(C)** Table summarising characteristics of human AD samples.

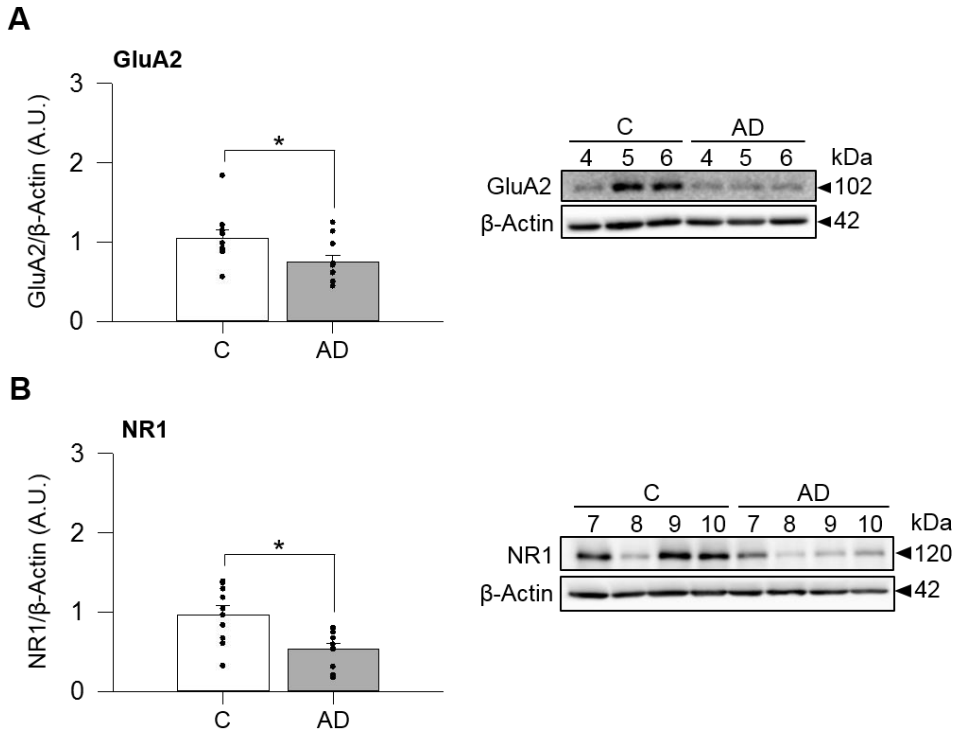


Figure 4-2. Ionotropic glutamate receptor protein expression in *post-mortem* AD samples. (A) GluA2 protein levels are significantly reduced in the Alzheimer's disease (AD) group (N = 10) compared to the control (C) group (N = 10). **(B)** NR1 protein levels are significantly reduced in the AD group (N = 10) compared to the C group (N = 10). Circles represent the value for each subject and error bars represent the standard error of the mean (S.E.M.). Significance level (*p < 0.05) was determined by using an unpaired Student t-test. A.U.: Arbitrary units. Insets show representative immunoblots. For technical purposes, samples were loaded in numerical order to facilitate the work, but not due to scientific reasons. See **Appendix** for examples of full-length immunoblots for each antibody and for immunoblots corresponding to the rest of the samples.

Changes in protein levels of group I metabotropic glutamate receptors, mGluR1 and mGluR5, were then examined. Quantification of mGluR1 protein amounts showed no differences between control and AD groups (C: 0.89 ± 0.08, AD: 0.97 ± 0.08, p-value = 0.457, **Figure 4-3A**). On the contrary, mGluR5 protein levels were markedly decreased in AD brains compared to controls (C: 1.72 ± 0.22, AD: 0.85 ± 0.15, p-value = 0.004, **Figure 4-3B**). These results suggest that AD pathology specifically affects mGluR5, whereas protein levels of a closely related receptor, mGluR1, remain unaffected. Since data from **Chapter 3** showed the mGluRs-mediated inhibition of mAChRs, and a previous study has outlined that mGluR5 can negatively impact on mAChRs (Jo et al. 2006), it was possible that decreased mGluR5 levels would lead to an alteration in mAChRs levels. Therefore, protein amounts of mAChR1 were evaluated by western blot. This analysis showed no

significant changes in protein levels between control and AD brains (C: 1.06 ± 0.16, AD: 1.03 ± 0.17, p-value = 0.915, **Figure 4-3C**).

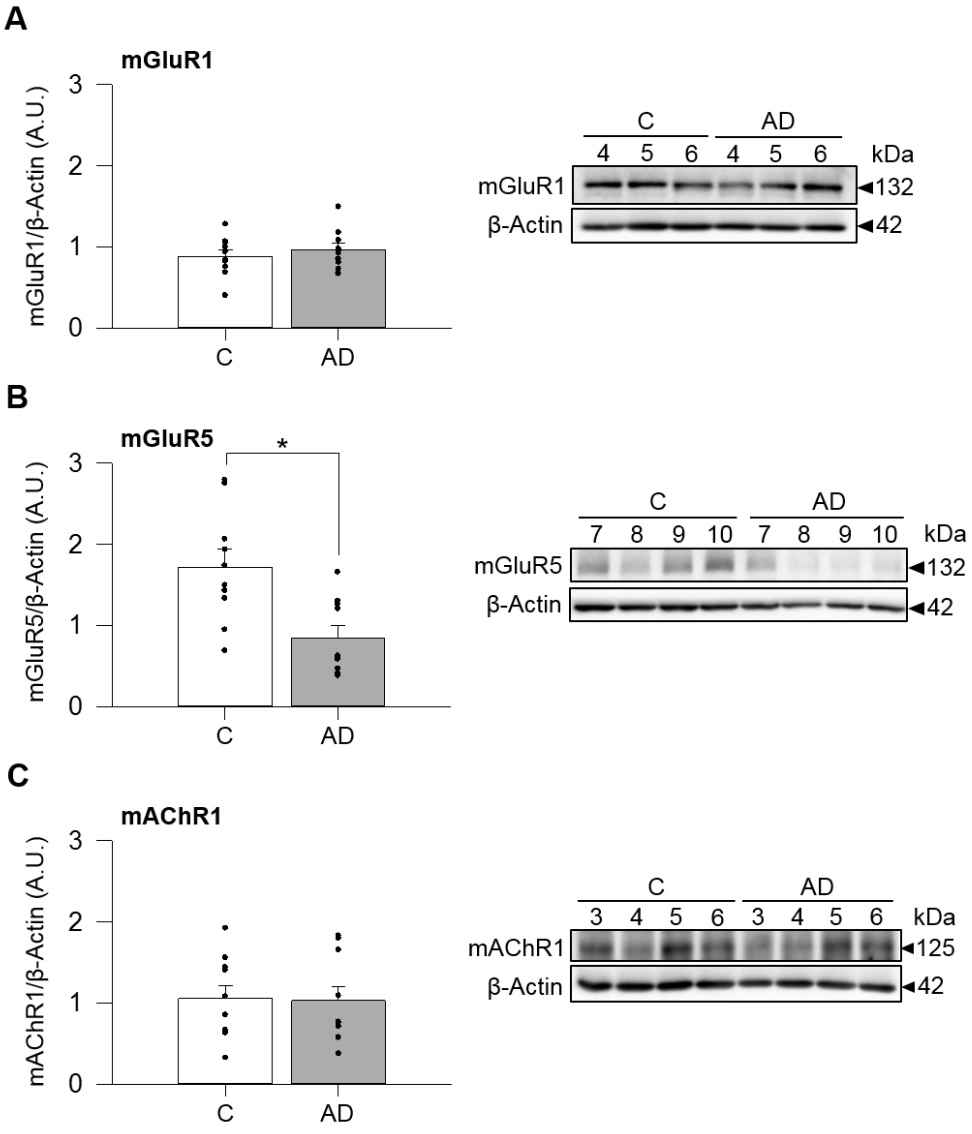


Figure 4-3. Synaptic GPCR protein expression in *post-mortem* AD samples. (A) mGluR1 protein levels are not significantly different in the Alzheimer's disease (AD) group (N = 10) compared to the control (C) group (N = 10). **(B)** mGluR5 protein levels are significantly reduced in the AD group (N = 10) compared to the C group (N = 10). **(C)** mAChR1 protein levels are not significantly different in the AD group (N = 10) compared to the C group (N = 10). Circles represent the value for each subject and error bars represent the standard error of the mean (S.E.M.). Significance level (*p < 0.05) was determined by using an unpaired Student t-test. A.U.: Arbitrary units. Insets show representative immunoblots. For technical purposes, samples were loaded in numerical order to facilitate the work, but not due to scientific reasons. See **Appendix** for examples of full-length immunoblots for each antibody and for immunoblots corresponding to the rest of the samples.

The location and signalling of mGluR5 is affected by its interaction with Homer 1 b/c, a scaffold protein located in the postsynaptic density (Roche et al. 1999; Tu et al. 1999; Sergé et al. 2002; Mao et al. 2005). In addition, Homer 1b/c and mGluR5 interaction can be disrupted by Aβ pathology, having pathological consequences (Roselli et al. 2009; Ronesi et al. 2012; Haas et al. 2016). Therefore, a decrease in the levels of mGluR5 could be associated with a decrease in Homer 1b/c levels. Western blot analysis with Homer 1b/c antibody showed that Homer 1b/c protein levels were significantly decreased in AD brains compared to controls (C: 0.72 ± 0.07, AD: 0.37 ± 0.05, p-value = 0.001, **Figure 4-4A**). Since this decrease was detected in the same AD samples that showed a reduction in mGluR5 protein levels, this suggests that reduced Homer 1b/c levels may be related to the reduced expression of mGluR5.

Finally, changes in protein expression levels may be explained by an overall loss of neurons and/or synapses, which can be estimated by measuring levels of synaptic markers. To measure postsynaptic changes, an antibody against postsynaptic density 95 (PSD95) was used. PSD95 is an integral scaffold protein located in the postsynaptic region of synapses that is classically used as a postsynaptic marker (Dorostkar et al. 2014; Kurbatskaya et al. 2016). Analysis of immunoblots incubated with PSD95 antibody revealed no significant differences in protein expression between AD and control groups (C: 1.50 ± 0.18, AD: 1.11 ± 0.18, p-value = 0.121, **Figure 4-4B**). Since synapses are composed of pre- and postsynaptic elements and presynaptic inputs are necessary for clustering of glutamate receptors in the postsynaptic element (Rao et al. 2000), changes in presynaptic terminals were assessed. To do this, an antibody against synaptophysin (SYP) was used. Synaptophysin is a glycoprotein found in presynaptic vesicles whose expression serves as a measurement of presynaptic abundance (Jahn et al. 1985; Wiedenmann & Franke 1985; Navone et al. 1986). Analysis of synaptophysin protein expression in AD patient samples showed no differences in protein levels between control and AD samples (C: 1.67 ± 0.19, AD: 1.33 ± 0.16, p-value = 0.192, **Figure 4-4C**). Altogether these results show that no loss of pre- and postsynaptic markers could be detected in temporal lobe samples from AD patient brains as measured by western blot analysis of total protein homogenates.

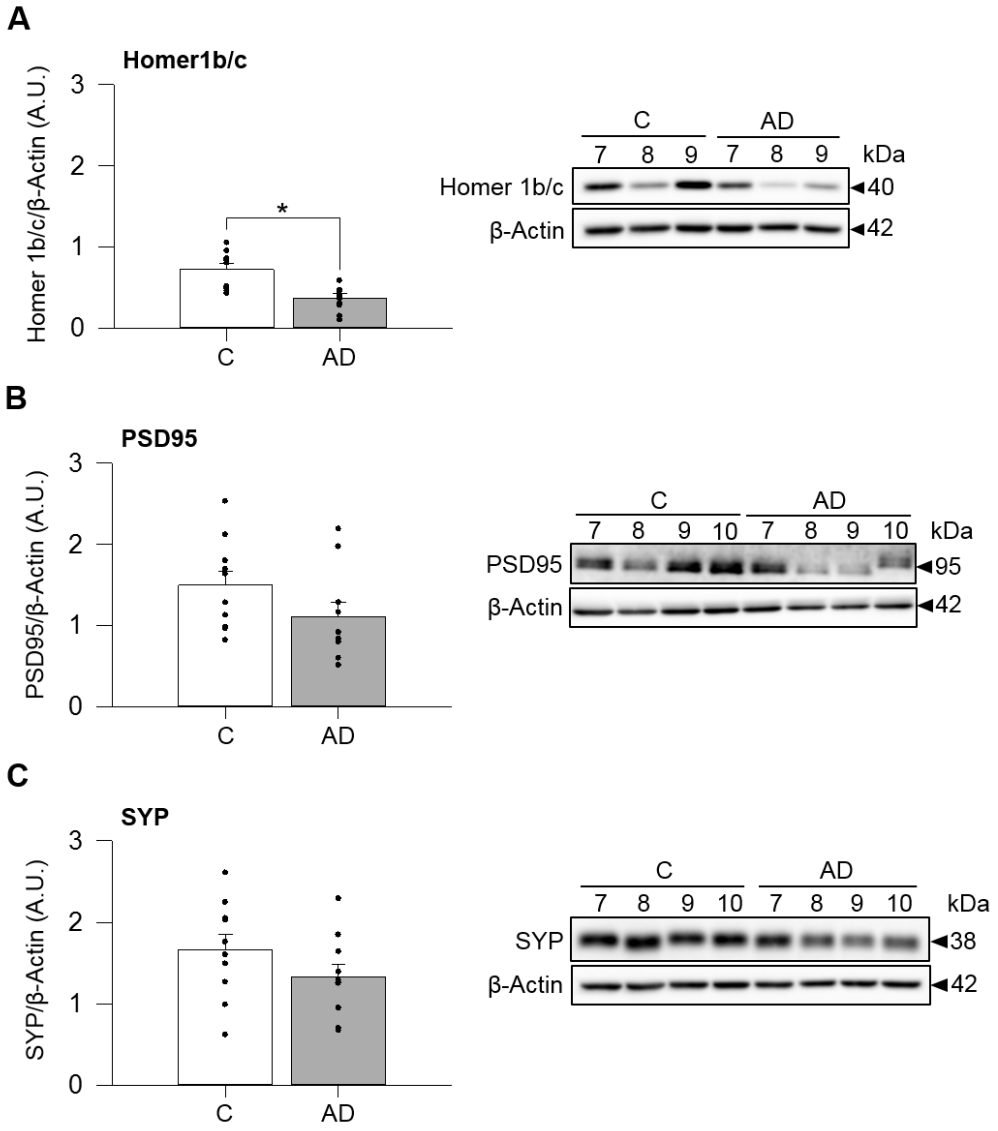


Figure 4-4. Synaptic markers protein expression in *post-mortem* AD samples. (A) Homer 1b/c protein levels are significantly reduced in the Alzheimer's disease (AD) group (N = 10) compared to the control (C) group (N = 10). **(B)** PSD95 (shift in band corresponding to sample AD10 is due to the gel being broken during the transfer step) and **(C)** SYP protein levels are not significantly different in the AD group (N = 10) compared to the C group (N = 10). Circles represent the value for each subject and error bars represent the standard error of the mean (S.E.M.). Significance level (*$p < 0.05$) was determined by using an unpaired Student t-test. A.U.: Arbitrary units. Insets show representative immunoblots. For technical purposes, samples were loaded in numerical order to facilitate the work, but not due to scientific reasons. See **Appendix** for examples of full-length immunoblots for each antibody and for immunoblots corresponding to the rest of the samples.

4.2.2 Co-immunoprecipitation between mGluR5 and Homer 1 b/c

Since a reduction in both mGluR5 and Homer 1b/c levels was observed, the study next aimed to determine whether their interaction was also affected in disease conditions. This would be relevant as Homer 1 b/c can influence mGluR5 location and function, as previously mentioned (Roche et al. 1999; Sergé et al. 2002; Mao et al. 2005). To do this, co-immunoprecipitation with protein samples from AD and control brain tissue was attempted. However, this presented a technical difficulty that could not be easily overcome. As shown in **Chapter 3** (see **section 3.2.4**), extraction of mGluR5 protein could only be achieved by the use of sucrose/SDS buffer. This is a very stringent buffer and the presence of SDS causes denaturing of proteins and hence can disrupt protein-protein interactions. However, in co-immunoprecipitation techniques, maintaining the interaction complex intact is a key requirement for successful detection of the interaction. For this reason, buffers containing non-denaturing components with low ionic strength are generally used in co-immunoprecipitation techniques (Thermo Scientific 2010). Therefore, the possibility of using sucrose/SDS buffer for protein extraction in the co-immunoprecipitation protocol was discarded. A buffer that was less likely to disrupt protein-protein interactions was used instead and co-immunoprecipitation was performed using an antibody against Homer 1b/c as the IP antibody. This resulted in no extraction of mGluR5 protein in the input fractions, although Homer 1 b/c could be detected in western blot (**Figure 4-5**). Due to the limitation imposed by the necessity of using a denaturing buffer to obtain mGluR5 bands by western blot, co-immunoprecipitation of mGluR5 and Homer 1b/c was not pursued further.

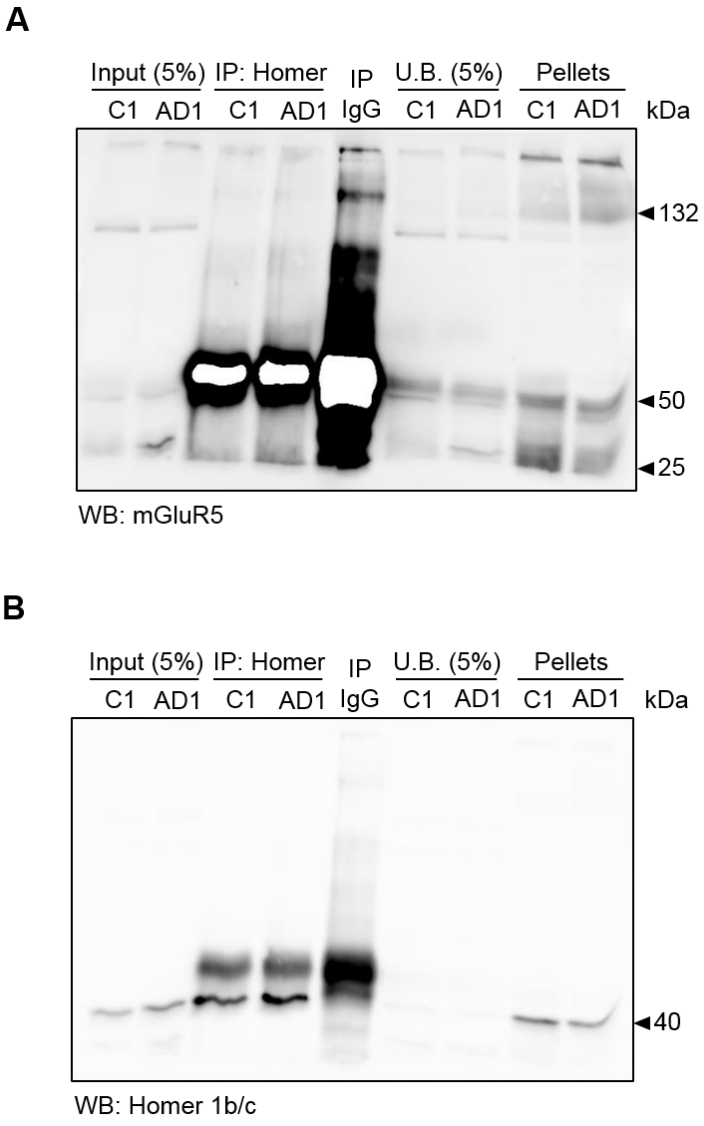


Figure 4-5. Co-immunoprecipitation of Homer 1b/c and mGluR5 in *post-mortem* AD samples. (A) Membrane incubated with anti-mGluR5 **(B)** Membrane incubated with anti-Homer 1b/c. Input sample refers to the lysate resulting after tissue homogenisation that is pre-cleared by incubation with protein-G agarose beads. Immunoprecipitation (IP) sample refers to the fraction taken from the input tube that is incubated with the pull-down antibody, in this case anti-Homer, or the control antibody, anti-IgG. Unbound fraction (U.B.) refers to the fraction of the IP sample that has not bound to the antibody. Pellet refers to the material accumulated at the bottom of the tube after tissue homogenisation. Western blot (WB) antibody indicates the antibody used for immunoblotting. For details of the procedure see **Section 2.9**.

4.2.3 Gene expression of synaptic proteins in post-mortem human brain from AD patients

Following the results of protein expression in human AD brains, the study aimed to investigate the causes of mGluR5 decrease. One plausible cause for this decrease is a reduction in mGluR5 transcripts, which may result in a decrease in protein

translation (Bauernfeind & Babbitt 2017). To measure gene expression levels, quantitative polymerase chain reaction (qPCR) was used. This is a very sensitive technique for which the identification of valid endogenous controls for data normalisation is essential (Coulson et al. 2008). Therefore, the first step was to identify an appropriate endogenous control gene in the set of *post-mortem* human brain samples. This selection was made according to (Rydbirk et al. 2016) and resulted in three candidate endogenous genes: ACTB (β-actin), GUSB (β-Glucuronidase) and ribosomal protein large 13 (RPL13). qPCR analysis of these transcripts confirmed that the most stably expressed gene across all samples was RPL13 (**Figure 4-6**). Therefore, this gene was selected as endogenous control for data normalisation in qPCR analysis. Next, levels of mGluR5 gene expression were analysed by qPCR. This showed that mGluR5 gene expression was significantly reduced in AD brain samples compared to control samples (C: 14.97 ± 9.85, AD: 0.005 ± 0.002, p-value = 0.010, **Figure 4-7A**). As a comparison to other postsynaptic proteins, levels of GluA2 and PSD95 gene expression were also measured. The levels PSD95 were also significantly decreased in AD brains (C: 0.07 ± 0.05, AD: 0.0 ± 0.01, p-value = 0.011, **Figure 4-7B**). Levels of GluA2 however were not significantly different between control and AD groups (C: 0.40 ± 0.17, AD: 0.20 ± 0.11, p-value = 0.186, **Figure 4-7C**). This data suggests that a reduction in gene expression levels of mGluR5 could account for a reduction in protein levels in AD brain samples. In the case of PSD95 and GluA2, changes in protein expression did not correlate with changes in gene expression, suggesting that post-translational modifications may affect the expression of these proteins.

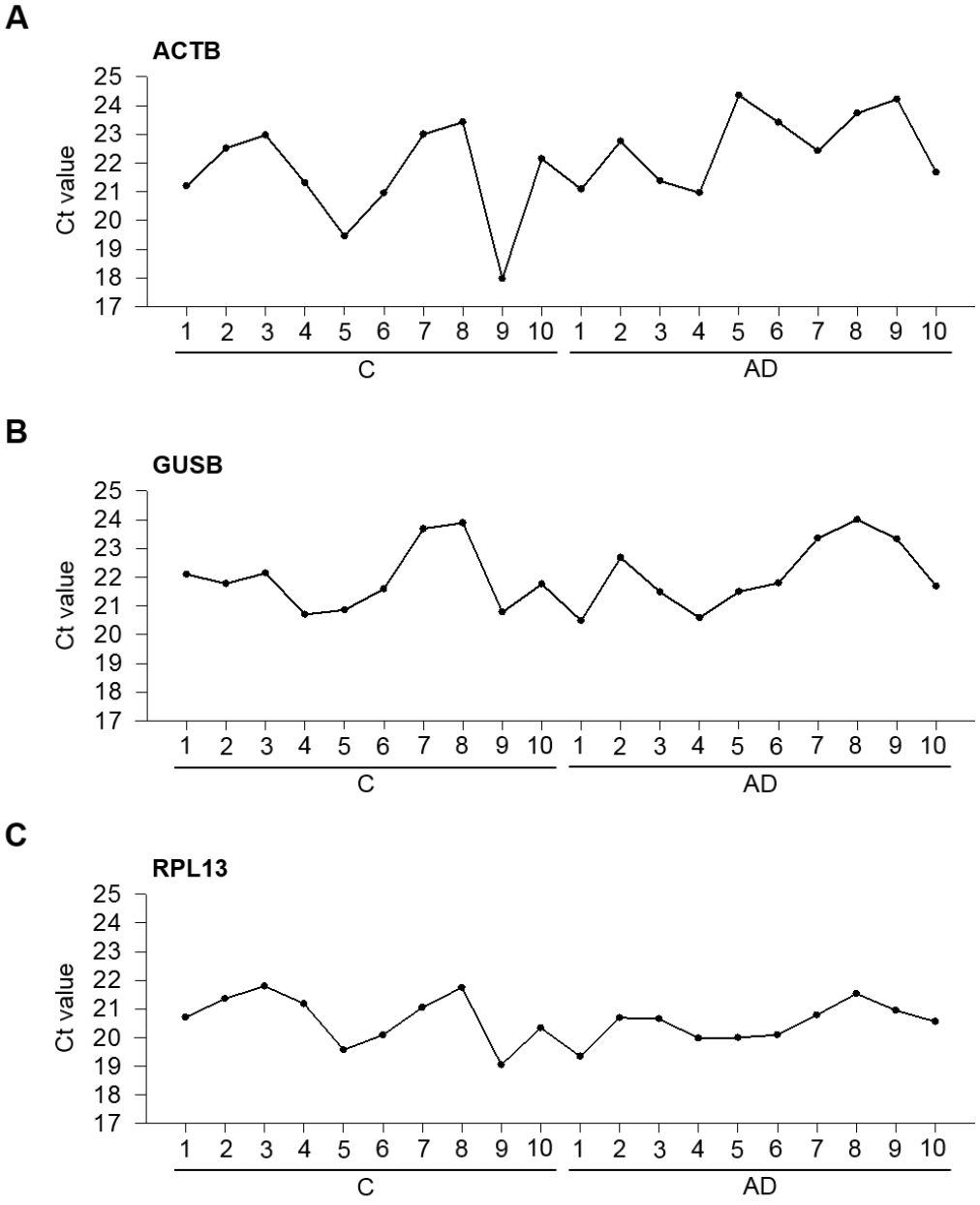


Figure 4-6. Gene expression of candidate endogenous control genes in *post-mortem* AD samples and age-matched control samples. Raw Ct values for **(A)** ACTB (β-actin), **(B)** GUSB (β-glucuronidase) and **(C)** RPL13 (ribosomal protein large 13).

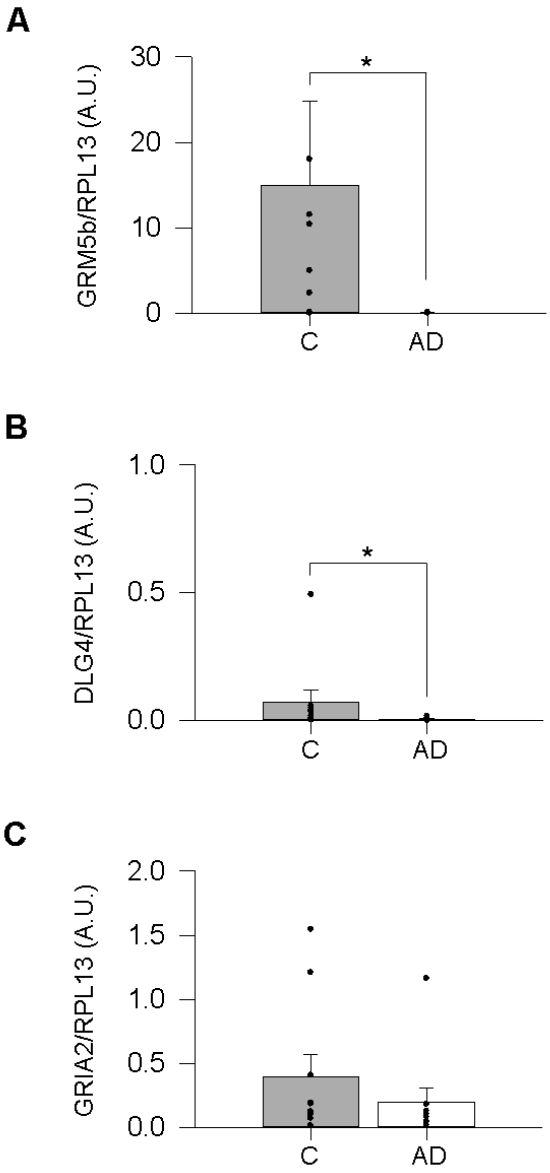
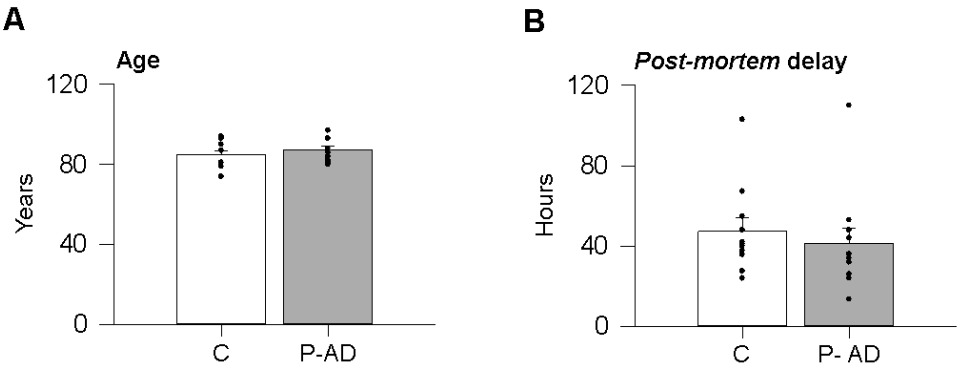


Figure 4-7. Gene expression in human *post-mortem* AD samples. Gene expression levels of **(A)** GRM5b (mGluR5b) and **(B)** DLG4 (PSD95) are significantly reduced in AD (N = 10) group compared to C (N =11) but not **(C)** GRIA2 (GluA2). Circles represent the mean and error bars the standard error of the mean (S.E.M.). Significance level (*p < 0.05) was determined by using a Mann-Whitney rank sum test. A.U.: Arbitrary units.

4.2.4 Synaptic protein expression in post-mortem human brain from P-AD patients

Given the progressive nature of neuropathological changes in AD, alterations in protein expression observed in severe AD cases may not be the same at earlier stages of the disease. Therefore, the same western blot analysis was undertaken in a set of samples from patients diagnosed with possible/probable AD (Braak stages III-IV) and aged-matched controls (Braak stages 0-III). Clinically, these

patients may or may not show manifestation of dementia as assessed by cognitive score systems but their neuritic plaque score suggests the diagnosis of AD, according to CERAD criteria (see **Chapter 2, section 2.3**). No differences in age (C: 85 ± 1.97, P-AD: 87.36 ± 2.00, p-value = 0.410, **Figure 4-8A**) or *post-mortem* delay (C: 47.36 ± 6.63, P-AD: 41.50 ± 7.63 p-value = 0.324, **Figure 4-8B**) were detected between control and P-AD groups, indicating that these were not likely to have an effect on further analysis.



C

Group	Age (mean)	Age (SEM)	PM delay (mean)	PM delay (SEM)	Females	Males	Total
C	85	2	47.4	6.6	6	5	11
P-AD	87.4	2	41.5	7.6	6	5	11

Figure 4-8. Age and *post-mortem* delay distribution of *post-mortem* P-AD samples. **(A)** No significant differences in age were found between the control (C) group (N = 11) and the probable/possible Alzheimer's disease (P-AD) group (N = 11) **(B)** No significant differences in *post-mortem* delay were found between the C group (N = 11) and the P-AD group (N = 11). Circles represent the value for each subject and error bars represent the standard error of the mean (S.E.M.). Significance level (*p < 0.05) was determined by using an unpaired Student t-test. **(C)** Table summarising characteristics of human P-AD samples.

Same pattern of analysis to that performed with AD samples was applied to P-AD samples. Western blot analysis of GluA2 levels showed a significant reduction in protein amounts in P-AD brain samples compared to control samples (C: 0.96 ± 0.07, P-AD: 0.65 ± 0.12, p-value = 0.042, **Figure 4-9A**). On the contrary, protein levels of NR1 we not significantly changed in the P-AD group compared to the control group (C: 0.82 ± 0.10, P-AD: 0.71 ± 0.13, p-value = 0.508, **Figure 4-9B**). This data suggests that NMDARs may be less affected than AMPARs at early stages of AD pathology.

Levels of mGluRs were then assessed. Western blot analysis showed no significant changes in protein levels of mGluR1 (C: 0.78 ± 0.08, P-AD: 0.80 ± 0.08,

p-value = 0.875, **Figure 4-10A**) and mGluR5 (C: 1.55 ± 0.39, P-AD: 0.88 ± 0.20, p-value = 0.139, **Figure 4-10B**) between the P-AD group and the control group. However, levels of the synaptic GPCR, mAChR1 were significantly reduced in the brains of P-AD patients compared to control subjects (C: 2.09 ± 0.25, P-AD: 0.82 ± 0.14, p-value = 0.001, **Figure 4-10C**). This data indicates that mAChR1 decreases at early stages of AD but that this decrease is not detected at late stages.

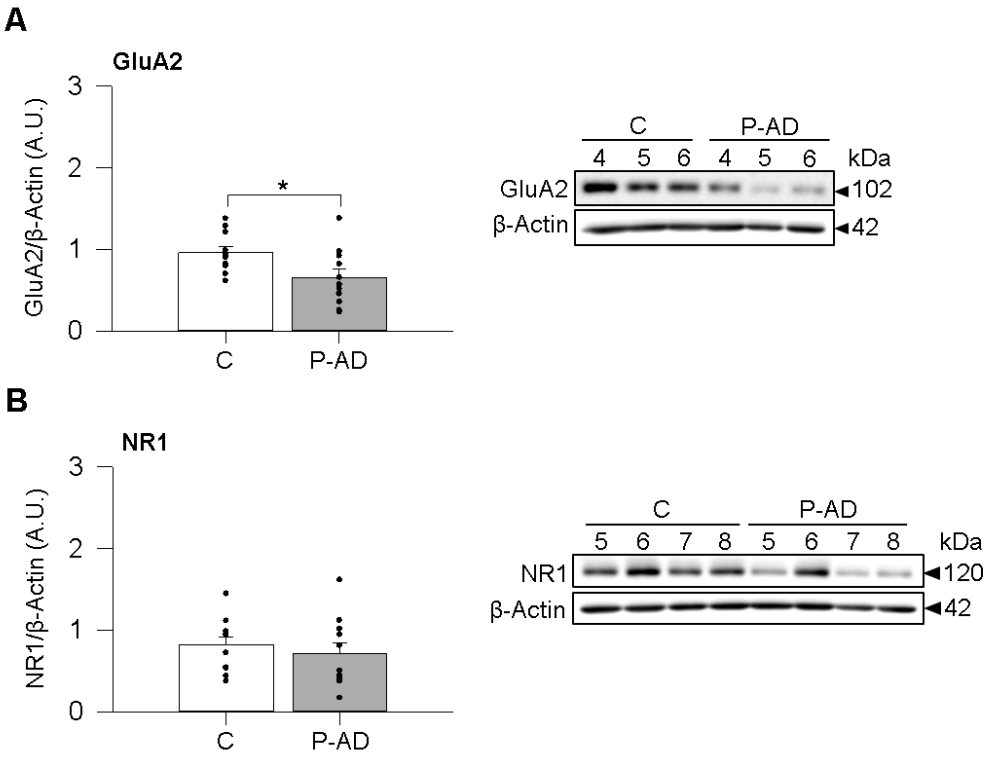


Figure 4-9. Ionotropic glutamate receptor protein expression in *post-mortem* P-AD samples. (A) GluA2 protein levels are significantly reduced in the probable/possible Alzheimer's disease (P-AD) group (N = 11) compared to the control (C) group (N = 11). **(B)** NR1 protein levels are not significantly different in the P-AD group (N = 11) compared to the C group (N = 11). Circles represent the value for each subject and error bars represent the standard error of the mean (S.E.M.). Significance level (*p < 0.05) was determined by using an unpaired Student t-test. A.U.: Arbitrary units. Insets show representative immunoblots. For technical purposes, samples were loaded in numerical order to facilitate the work, but not due to scientific reasons. See **Appendix** for examples of full-length immunoblots for each antibody and for immunoblots corresponding to the rest of the samples.

As in the case of the previous analysis for the set of AD samples, loss of synaptic terminals was assessed to check whether it could account for changes in protein expression. Homer 1b/c protein levels did not differ significantly between P-AD and control brains (C: 0.79 ± 0.07, P-AD: 0.56 ± 0.12, p-value = 0.10, **Figure 4-11A**).

This was also the case for protein levels of postsynaptic marker PSD95 (C: 1.04 ± 0.13, P-AD: 0.90 ± 0.17, p-value = 0.529, **Figure 4-11B**) as well as for the presynaptic marker SYP (C: 1.34 ± 0.11, P-AD: 1.06 ± 0.18, p-value = 0.187, **Figure 4-11C**). These results suggest that in the samples analysed in these study, synaptic marker protein expression is not significantly altered at any stage of AD pathology.

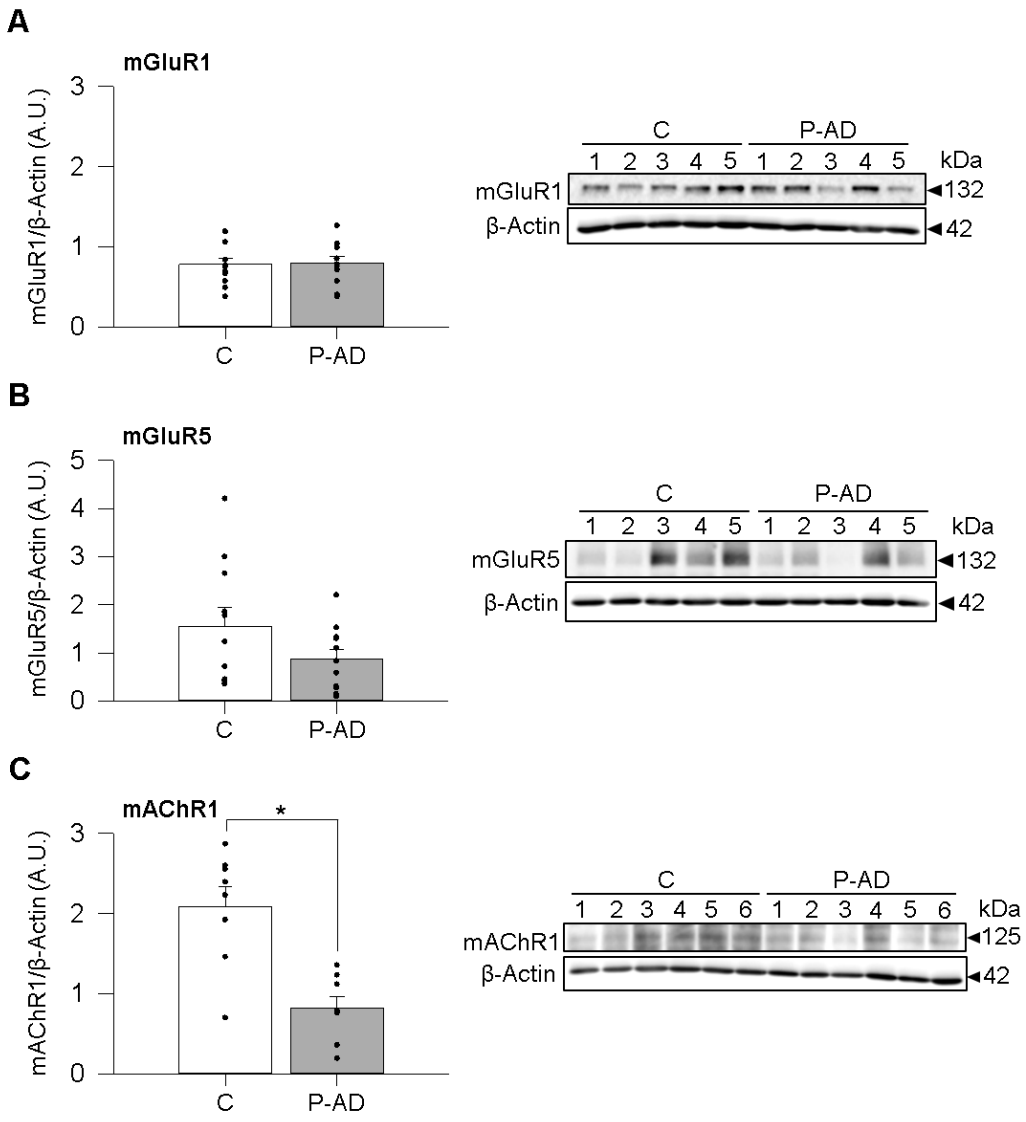


Figure 4-10. Synaptic GPCR protein expression in *post-mortem* P-AD samples. (A) mGluR1 protein levels are not significantly different in the probable/possible Alzheimer's disease (P-AD) group (N = 11) compared to the control (C) group (N = 11). **(B)** mGluR5 protein levels are not significantly different in the P-AD group (N = 11) compared to the C group (N = 11). **(C)** mAChR1 protein levels are significantly reduced in the P-AD group (N = 8) compared to the C group (N = 8). Circles represent the value for each subject and error bars represent the standard error of the mean (S.E.M.). Significance level (*p < 0.05) was determined by using an unpaired Student t-test. A.U.: Arbitrary units. Insets show representative immunoblots. For technical purposes, samples were loaded in numerical

order to facilitate the work, but not due to scientific reasons. See **Appendix** for examples of full-length immunoblots for each antibody and for immunoblots corresponding to the rest of the samples.

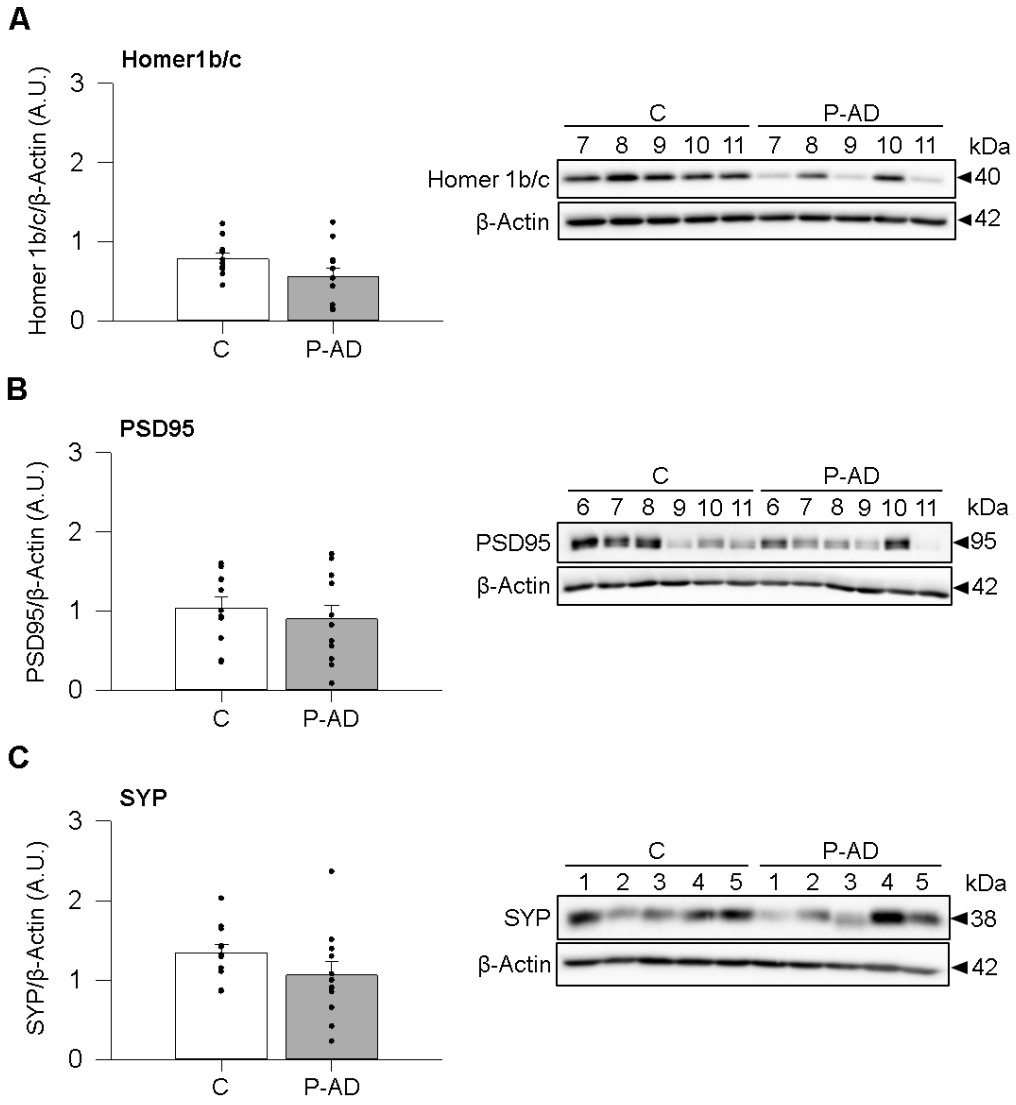


Figure 4-11. Synaptic markers protein expression in *post-mortem* P-AD samples. (A) Homer 1b/c, **(B)** PSD95 and **(C)** SYP protein levels are not significantly different in the probable/possible Alzheimer's disease (P-AD) group (N = 11) compared to the control (C) group (N = 11). Circles represent the value for each subject and error bars represent the standard error of the mean (S.E.M.). Significance level (*$p < 0.05$) was determined by using an unpaired Student t-test. A.U.: Arbitrary units. Insets show representative immunoblots. For technical purposes, samples were loaded in numerical order to facilitate the work, but not due to scientific reasons. See **Appendix** for examples of full-length immunoblots for each antibody and for immunoblots corresponding to the rest of the samples.

4.3 Discussion

In this study, the expression of a select group of synaptic proteins relevant to AD was analysed in *post-mortem* temporal lobe samples from patients diagnosed with definite AD and probable/possible AD (P-AD), according to CERAD principles (see **Chapter 2, section 2.3**). Considering data from **Chapter 3** showing an mGluRs-mediated inhibition of mAChRs function, it was of interest to assess whether the expression of these receptors was affected in AD, as it could have functional implications for pathological mechanisms. In addition, other synaptic proteins analysed included ionotropic glutamate receptors, to evaluate how the glutamatergic system is affected in AD, and synaptic markers, as they are good indicators of the state of synapses.

First, the levels of ionotropic glutamate receptors were analysed. A significant reduction in GluA2 and NR1 proteins in AD brains (**Figure 4-2**) suggested that GluA2-containing AMPARs and NR1-containing NMDARs are affected at late stages of AD pathology. In the case of GluA2, this decrease seemed to start at earlier stages of the pathology as protein levels were also reduced in samples from P-AD patients (**Figure 4-9A**).

Presumably, protein extraction performed in this study reflects total protein levels, including protein expressed intracellularly and at the plasma membrane. Therefore, the reduction in GluA2 levels observed here corresponds to overall protein levels. Interestingly, it has been shown that a reduction in AMPARs expressed at postsynaptic membranes is driven by Aβ (Hsieh et al. 2006; Shankar et al. 2007) and tau (Hoover et al. 2010) in *in vitro* models of AD. Since both pathologies are present in the AD brains studied here, it is reasonable to think that the reduction observed in this study may reflect a reduction in GluA2-containing AMPARs expressed at the plasma membrane. This could occur by increased receptor internalization or disruption of receptor trafficking to the membrane (Gu et al. 2009; Jurado et al. 2018). However, because protein internalised to endocytic vesicles would also be included in the analysis here, the reduction in overall GluA2 levels may reflect a reduction in its synthesis or an increased degradation of the receptor. The former seems not to be the case, at least at late stages of AD, as GluA2 mRNA levels were not significantly changed in AD samples compared to controls (**Figure 4-7C**). Degradation of AMPARs is governed by numerous factors that may be relevant to this study. For instance, GluA2-containing AMPARs are

degraded in the proteasome as a result of reduced activity of sodium-potassium ATPase (Zhang et al. 2009). A reduction in activation of sodium-potassium ATPase activity and expression has been previously shown to occur in AD brains (Chauhan et al. 1997; Hattori et al. 1998) and could be driven by Aβ present in these brains (Petrushanko et al. 2016; Ohnishi et al. 2015). In addition, proteasomal degradation of GluA2-containing AMPARs requires NMDAR activity (Hou et al. 2011) and it is specifically regulated by activity-dependent sorting mechanisms (Lussier et al. 2011; Lee et al. 2004). Supporting that NMDAR activity is a strong regulator of AMPARs degradation, it has been shown that NMDARs activation selectively diverts GluA2 from the recycling pathway toward degradation via lysosomal pathway (Lee et al. 2004). Since an increase in NMDARs activity (Texidó et al. 2011; Alberdi et al. 2010; Molnár et al. 2004) and a decrease in sodium-potassium ATPase activity (Wei et al. 2016; Hattori et al. 1998) have been shown to occur in AD-related pathology, it is plausible that the combination of both mechanisms operating in the brain samples studied here contributes to the reduced levels of GluA2 observed via enhanced degradation. In addition, other studies showed that loss of GluA2 subunits was not due to neuronal loss in the entorhinal cortex of AD patients by immunohistochemical and western blot techniques (Armstrong et al. 1994; Yasuda et al. 1995). It has also been suggested that loss of GluA2 subunits may be an early sign of AD pathology as it was shown to occur prior to neuronal loss (Armstrong et al. 1994) and NFT formation (Ikonomovic et al. 1997). This is supported by a study showing loss of GluA2 immunoreactivity in brains from AD patients at Braak stages III-IV (Ikonomovic et al. 1997) as well as by results presented here showing a reduction in GluA2 levels occurring at early stages of the pathology (in P-AD brain samples) (**Figure 4-9A**).

The presence of GluA2 subunits in the AMPAR complex substantially reduces calcium permeability of the receptor (Hollmann et al. 1991). Given the role of calcium excitotoxicity in the pathological mechanisms of AD (LaFerla 2002), it has been proposed that GluA2-lacking calcium permeable AMPARs (CP-AMPARs) may play key roles in some of these mechanisms (Whitehead et al. 2017). Indeed, increased expression of CP-AMPARs has been reported in AD transgenic mice (Megill et al. 2015). A role of CP-AMPAR in AD is further supported by the finding that intracellular administration of Aβ oligomers in CA1 hippocampal neurons results in the insertion of CP-AMPARs in the plasma membrane (Whitcomb et al. 2015). Importantly, intracellular accumulation of Aβ has been reported in AD brains

(Wegiel et al. 2007; Aoki et al. 2008; Nagele et al. 2002; D'Andrea et al. 2002) and this seems to be an early event of the pathology as it precedes formation of Aβ plaques and NFTs (D'Andrea et al. 2001; Gouras et al. 2000). Therefore, the reduction in GluA2 levels reported in the present study is consistent with a mechanism involving a pathological increase in CP-AMPARs starting at early stages of disease progression.

While the reduction in GluA2-containing AMPARs started markedly at early stages of AD, this was not the case for the other group of ionotropic glutamate receptor analysed, as NR1-containing NMDARs were only reduced at late stages (**Figure 4-2B** and **Figure 4-9B**). This result is in agreement with findings of others (Sze et al. 2001; Ikonomovic et al. 1999; Hynd et al. 2004b), and suggests that at early stages of AD, NMDAR protein expression is somehow maintained at similar levels than in control brains. It has been proposed that this may occur by virtue of compensatory mechanisms, including dendritic sprouting (Masliah et al. 1991). This involves the growth of dendritic spines into denervated areas, a process shown to occur in presenile dementia (Scheibel & Tomiyasu 1978). Therefore, NMDARs levels may be maintained at early stages of AD pathology by action of dendritic sprouting mechanisms that eventually cannot counterbalance the loss of NMDARs at late stages.

Another proposed mechanism of maintaining NMDAR levels in P-AD brains is the upregulation of these receptors by specific phosphorylation at this stage of the pathology (Raymond et al. 1994; Sze et al. 2001; Farber et al. 1998; Olney et al. 1997). This is in line with the well-established feature of AD pathology consisting of increased NMDAR function (Vosler et al. 2008; Alberdi et al. 2010; Kelly & Ferreira 2006; Harkany et al. 2000). Accordingly, data presented here could be explained by an initial upregulation of NMDAR function at early stages of pathology. Then, cumulative overactivation of NMDARs by glutamate built up during the progression of AD could eventually lead to Aβ-induced endocytosis of NMDARs (Snyder et al. 2005) and reduction of overall levels at late stages. In addition, because NR1-expressing cells may be selectively vulnerable to toxicity associated with AD (Hynd et al. 2004b), the effect of this vulnerability could become more evident as neurodegeneration progresses and neurons die. This could eventually lead to a significant overall loss of NR1-containing cells at late stages, when neurons can no longer face neurotoxicity. The possibility that reduced

translation of NR1 mRNA underlies the reduction in protein levels cannot be discarded. However, the correlation between changes in NR1 mRNA and protein levels in AD brains seems to be variable depending on the brain areas (Panegyres et al. 2002). Nonetheless, the findings presented here are relevant to the clinical impact of AD pathology since the severity of alterations in NR1 subunits seemed to strongly correlate with the magnitude of cognitive impairments associated with AD (Sze et al. 2001). This is not surprising given that the molecular mechanism thought to underlie memory processes relies to a great extent on the function of NMDARs (Martin et al. 2000; Lissin et al. 1998; Lu et al. 2001; Shi et al. 1999; Malenka 2003), for which the presence of NR1 subunits is required (Wang et al. 2009).

In addition to ionotropic glutamate receptors, the glutamatergic system includes mGluRs, which are also mediators of AD-related neuropathological mechanisms (Revett et al. 2013). Radioligand binding studies have shown a general reduction in the levels of mGluRs in the hippocampus and cortex of AD patients (Dewar et al. 1991; Albasanz et al. 2005). This is in agreement with results presented here showing a specific reduction of mGluR5 but not mGluR1 levels in the brains of AD patients (**Figure 4-3** and **Figure 4-10**).

The reduction in mGluR5 protein levels is entirely consistent with the finding that mGluR5 gene expression is downregulated in brains of AD patients (**Figure 4-7A**). Therefore, it seems plausible that a reduction in mGluR5 transcription contributes, at least in part, to the observed reduction in protein levels. Additionally, it is well-described that chronic glutamate exposure can lead to down-regulation and desensitization of mGluRs (Albasanz et al. 2005; Doherty et al. 1999; Catania et al. 1991). Since glutamate clearance mechanisms may be impaired in AD brains (Jacob et al. 2007; Scott et al. 2011; Masliah et al. 1996; Li et al. 1997), it is possible that the levels of glutamate in these brains are very high. This could lead to an excessive activation of mGluR5, a process that has been reported to play a role in AD-related pathologies (Hsieh et al. 2006; Hamilton et al. 2016; Hamilton et al. 2014; Shankar et al. 2008; Wang 2004; Um et al. 2013; Bruno et al. 2000). This in turn could result in its desensitisation and reduced levels at the plasma membrane (Dhami & Ferguson 2006; Albasanz et al. 2005). Furthermore, internalisation could lead to increased receptor degradation. Proteasomal degradation of mGluR5 is induced by Siah1A (Moriyoshi et al. 2004) and a complex including Siah1A has

been shown to alter its location in brains from AD patients (Wasik et al. 2013). Therefore, it is possible that the rate of mGluR5 degradation is increased in AD brains as a result of dysregulation of degradation mechanisms.

Considering results from **Chapter 3** showing an inhibition of mAChR function by agonism of group I mGluRs, the reduction in mGluR5 levels could influence other synaptic proteins, including mAChR and its expression in pathology. It has been reported that a decrease in cholinergic projections and activity of cholinergic enzymes occurs in individuals with AD (Davies & Maloney 1976; Bowen et al. 1982; Quirion et al. 1986; Davis et al. 1999). Moreover, reduction in the coupling between mAChR1 receptors and G-proteins seems to happen in AD brains (Shiozaki & Iseki 2004; Smith et al. 1987; Flynn et al. 1991; Warpman et al. 1993; Ferrari-DiLeo et al. 1995). However, whether these effects are due to a reduction in mAChR1 levels is not clear yet. In the present study, a prominent reduction of mAChR1 levels was detected at earlier stages of the pathology but no significant changes were found at late stages (**Figure 4-3C** and **Figure 4-10C**). These results are in agreement with evidence showing unchanged muscarinic binding in AD brains (Araujo et al. 1988; Svensson et al. 1992) and indicate that mAChR1 receptors are firstly affected by pathological mechanisms occurring in AD. Since this reduction seems to occur relatively early in the progression of the disease, it is possible that neurons are still healthy enough to activate compensatory mechanisms to counterbalance this loss at late stages. Given the severity of the loss of cholinergic afferents and enzymes in AD (Perry et al. 1977; Spillane et al. 1977; White et al. 1977), it has been proposed that neurons may respond by upregulating mAChRs levels (Nordberg et al. 1983). Indeed, upregulation of receptor expression as a response to afferent depletion has been reported (Levey 1995), indicating that is a plausible response in AD. This is supported by a prominent increase in mRNA levels of mAChR1 in the brains of AD patients, which may indicate that levels of protein also increase during disease (Harrison et al. 1991). In addition, compensatory increases in the levels of choline acetyltransferase (ChAT), the enzyme that synthesises ACh, have been reported in the brains of patients with MCI (DeKosky et al. 2002). Furthermore, it has been shown that destruction of the performant path in animal models results in sprouting of cholinergic afferents (Lynch et al. 1972; Storm-Mathisen 1974; Cotman et al. 1973; Savaskan & Nitsch 2001). This is consistent with reinnervation of acetyl cholinesterase (AChE) terminals in the hippocampus of AD brains in response to disease triggered cellular damage in the

entorhinal cortex (Hyman et al. 1987). Therefore, data presented here supports that after an initial loss of mAChR proteins, the cholinergic system may be capable of compensatory responses to counterbalance the consequences of neurodegeneration, which presumably manifest at later stages of the pathology.

Finally, an obvious reason for the decrease in receptor levels observed here is an overall decrease in synaptic terminals. To estimate the extent of loss of synaptic terminals in the samples analysed here, changes in synaptic markers were assessed. Western blot analysis of brain samples with an antibody against the postsynaptic marker PSD95 revealed no significant changes at any stage of AD progression (**Figure 4-4B** and **Figure 4-11B**). This agrees with results from a study that showed no differences in PSD95 levels by western blot analysis of association neocortex samples from AD subjects (Gylys et al. 2004). Regarding presynaptic terminals, no significant changes were detected in the levels of presynaptic marker synaptophysin in the AD or P-AD groups compared to controls (**Figure 4-4C** and **Figure 4-11C**), in agreement with other studies (Tannenberg et al. 2006; Harigaya et al. 1996; Hatanpää et al. 1999). Taken together these results indicate there is no significant loss of synaptic terminals in the samples analysed in this study. This may seem surprising considering that loss of synaptic connections has been widely correlated with early cognitive deficits in AD (Terry et al. 1991; DeKosky & Scheff 1990; Sze et al. 1997). However, this has not been consistently linked to a decrease in synaptic markers. For example, a significant increase in PSD95 in AD frontal cortex was reported (Leuba et al. 2008) which was also observed at the level of the postsynaptic density fraction (Gong et al. 2009). In the case of synaptophysin, levels were shown to be both reduced in the frontal cortex of early-AD brains (Masliah et al. 2001) and increased in frontal and temporal cortices (Mukaetova-Ladinska et al. 2000). In relation to this, a detailed analysis of *post-mortem* studies has shown that the degree to which changes in synaptic markers affect different brain areas is variable (De Wilde et al. 2016), which may explain the heterogeneity in the results. These findings support the concept that synapse loss and loss of synaptic markers may not be equivalent in AD. In line with this, results presented here support that numbers of pre- and postsynaptic terminals are unchanged over the course of AD but synapses may still be affected. Indeed, it cannot be excluded that actual synaptic connections are lost although pre- and postsynaptic terminals are present. However, these results suggest that rather

than a structural loss of synaptic connections, weakening of synaptic function may arise from loss of synaptic receptors in AD.

In summary, results from this chapter have shown that the expression of selected synaptic receptors is affected during AD progression. From a therapeutic perspective, it is very useful to know whether pathological changes observed in severe cases of AD occur at early stages of the pathology. This is because it is widely believed that treatments should be administered at early stages of pathology to be most effective. In this regard, this study has provided new insights into identifying receptors that are firstly affected in AD. In addition, since data from **Chapter 3** showed a functional interaction between mGluRs and mAChRs and these synaptic GPCRs are selectively affected in AD brains, the study next aimed to assess the functional consequences of AD pathology on these receptors. Therefore, **Chapter 5** is focused on the effects of human hyperphosphorylated tau, a hallmark of AD brains, on mGluRs and mAChRs function.

Chapter 5 Effect of tau pathology on synaptic GPCRs function

5.1 Introduction

5.2 Results

5.2.1 CCh-induced holding current change is increased in P-hTau transfected neurons

5.2.2 CCh-induced holding current change is not altered in WT-hTau transfected neurons

5.2.3 CCh-induced holding current change is not altered in AT8E-hTau transfected neurons

5.2.4 DHPG-induced holding current change is not altered in P-hTau transfected neurons

5.2.5 DHPG-induced holding current change is not altered in WT-hTau transfected neurons

5.3 Discussion

5.1 Introduction

Since the identification of tau as a central component of neurofibrillary tangles (NFTs) in AD brains (Kosik et al. 1989; Grundke-Iqbal, Iqbal, Quinlan, et al. 1986) and the importance of these structures in defining the neuropathology of the disease (Braak & Braak 1991), tau pathology has been in the spotlight of AD research for many years. Importantly, the so called "tauopathy" correlates strongly with the extent of cognitive decline (Nelson et al. 2012) and it is common to several neurodegenerative disorders, including frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17) and Pick's disease (PiD), among others (Williams 2006). This indicates that tauopathy is a critical feature of neurogenerative processes.

Tau was first characterised as a microtubule binding protein with a clear function in promoting and stabilising microtubule assembly (Drechsel et al. 1992; Weingarten et al. 1975). This function is intrinsically linked to the role of tau in axonal transport (Dixit et al. 2008) and elongation through the establishment of neuronal polarity (Caceres & Kosik 1990; Esmaeli-Azad et al. 1994). The gene that codes for tau, MAPT, contains 16 exons that are transcribed into a pre-mRNA (Andreadis et al. 1992). This pre-mRNA undergoes alternative splicing to generate six tau protein isoforms, all of them expressed in the human central nervous system (CNS) and raging from 352 to 441 amino acids (Drubin et al. 1984; Himmler 1989; Himmler et al. 1989; Kosik et al. 1989; Andreadis et al. 1992). Tau proteins contain a microtubule-binding domain that includes a proline-rich region, a C-terminal flanking region and at least three amino acid repeat regions, although some tau isoforms contain four repeats (Lee et al. 1989; Lee et al. 1988; Goedert, Spillantini, Potier, et al. 1989). In the N-terminus there is a projection domain that contains amino acid sequences coded by exon 2 or by exons 2 and 3. Therefore, depending on the number of N-terminal inserts and C-terminal repeats, six possible combinations give name to the six tau isoforms existing in the human brain: 0N3R, 0N4R, 1N3R, 1N4R, 2N3R and 2N4R (full-length) tau (Goode et al. 2000). These isoforms differ in their expression across the CNS which may be linked to differential susceptibility of brain regions to tau pathology in AD (Boutajangout et al. 2004).

The function of tau is highly regulated by its phosphorylation state which influences its ability to bind microtubules (Biernat et al. 1993; Lindwall & Cole 1984) and other

interacting partners and to self-aggregate (A. del C. Alonso et al. 2001). Thus, hyperphosphorylated tau disassembles from microtubules and aggregates in paired helical filaments (PHFs), one of the main pathological hallmarks in AD brains (Grundke-Iqbal, Iqbal, Tung, et al. 1986; Iqbal et al. 1986; Grundke-Iqbal, Iqbal, Quinlan, et al. 1986; Goedert et al. 1992). One obvious consequence of this is the destabilisation of microtubules (Alonso et al. 1994; Li et al. 2007; Alonso et al. 1996) and the resulting impaired axonal transport and loss of dendritic spines (Thies & Mandelkow 2007). Additionally, abnormal phosphorylation of tau confers the protein resistance to proteolytic cleavage by proteases (Wang et al. 1995; Wang et al. 1996) which results in a slower turnover of pathological tau compared to that of normal tau (Poppek 2006). This is supported by evidence showing that the levels of tau in AD brain are increased compared to controls (Khatoon et al. 1992). Part of tau expressed in AD brains is located to neurofibrillary tangles (NFTs), a pathological feature of these brains (Crowther et al. 1992; Crowther et al. 1994; Crowther et al. 1989; Goedert 1993) that correlates with severity of cognitive impairments (Arriagada et al. 1992; Braak & Braak 1991). Tau within extracellular NFTs specifically binds to antibodies directed to phosphorylated epitopes (serine o threonine residues), including AT8 (S199/S202/T205), AT100 (T212/S214), and PHF-1 (S396/S404), which corroborates that tau has a phosphorylation signature that characterises AD pathology (Augustinack et al. 2002; Duka et al. 2013).

The role of P-tau on synaptic damage goes beyond the disruption of microtubule stability (Jadhav et al. 2015). For instance, it can affect the function of postsynaptic receptors and therefore synaptic transmission. In relation to this, it was shown that P-tau localises to dendritic spines where it impairs the function of NMDARs and AMPARs located at the postsynaptic membrane by reducing their trafficking or anchoring to the PSD (Hoover et al. 2010). Another study showed that tau is required for the trafficking of Fyn kinase to the postsynaptic compartment (Ittner et al. 2010). This is relevant to pathology because Fyn forms a complex with PSD95 and phosphorylates the NR2B subunit of NMDARs (Ittner et al. 2010). This increases NMDARs stability at the PSD and it has been proposed as a mechanism that increases NMDAR-triggered excitotoxicity (Ittner et al. 2010; Mondragón-Rodríguez et al. 2012). Calcium influx through NMDARs can in turn activate tau kinases such GSK3β (Lesort et al. 1999) and Cdk5 (Hernandez et al. 2009) that further increase tau phosphorylation (Revett et al. 2013). In addition to NMDARs,

tau can exert pathological effects on AMPARs function. Illustrating this, there is evidence that pathological forms of tau can reduce AMPARs surface expression in cultured neurons (Hoover et al. 2010; Yu et al. 2012) and in animal models of tauopathy (Kopeikina et al. 2013). These effects on ionotropic glutamate receptors function may be related to the disruption of synaptic plasticity mediated by aberrant tau (Polydoro et al. 2009; Fá et al. 2016). This has been supported by studies showing a preferential accumulation of phosphorylated tau in the dendritic compartment where plasticity occurs (Hoover et al. 2010; Zempel et al. 2010). Altogether, this evidence supports a critical role of pathological tau in the regulation of synaptic structure and function with implications for AD.

One essential characteristic for the neuropathological diagnosis of AD is the presence of P-tau-containing NFTs in the autopsied brains. Consequently, the progression of AD is measured according to the anatomical distribution of NFTs on *post-mortem* brain, following the Braak staging procedure (Braak & Braak 1991). In the present study, patient cases were sorted according to Braak staging and divided into two groups: P-AD (Braak stages III-IV) and AD (Braak stages V-VI). As shown in **Chapter 4**, changes in the protein expression of GPCRs were observed in these brains. Therefore, it was of interest to test something unknown to date: whether P-human tau (P-hTau), a hallmark of AD brains and a critical marker of neuropathological progression, could affect the function of these receptors. The hypothesis being that P-hTau can disrupt the function of synaptic GPCRs as it does with other synaptic receptors (i.e. AMPARs and NMDARs) (Hoover et al. 2010). In particular, a reduction in the levels of mAChRs was observed in P-AD brains, suggesting that these receptors are susceptible at earlier stages of pathology. Therefore, it seems reasonable to think that an early effect of P-hTau expression in the neuron may be the disruption of mAChRs function. In relation to this, the effects of early P-hTau expression have been characterised to some extent in animal models. These studies are based on the principle that P-hTau can be functionally relevant when it is not part of NFTs. Supporting this, inhibition of P-hTau delayed motor dysfunction but did not change NFT numbers in tau transgenic mice (Le Corre et al. 2006). In addition, accumulation of P-hTau appears to be an early event occurring before NFTs formation in a transgenic mouse model of tauopathy (Berger et al. 2007). Importantly, this early accumulation of P-hTau may specifically happen in postsynaptic sites, suggesting that P-hTau may alter synaptic function. Indeed, P-hTau sorting to dendritic spines

is thought to be an early pathological feature of tauopathies (Gendron & Petrucelli 2009) and one early event associated with P-hTau localisation to dendrites is the disruption of synaptic function, particularly the reduction of miniature EPSCs (mEPSCs) (Hoover et al. 2010). This study also demonstrated that dendritic P-hTau was associated with decreased expression of AMPARs and NMDARs, suggesting that these molecular changes may underlie tau-mediated synaptic dysfunction at early stages of tau accumulation (Hoover et al. 2010).

In summary, there is evidence that P-Tau can affect the function of AMPARs and NMDARs in AD models but the effects on other receptors important for synaptic function, such as GPCRs, remain unknown. Therefore, experiments presented in this chapter aim to fill this gap in our current knowledge and assess whether P-hTau affects the function of synaptic GPCRs whose expression is affected in AD pathology. According to the above evidence and previous data from this thesis showing a reduced expression of mAChR1 observed at early stages of AD, it was hypothesised that expression of P-hTau reduces mAChR-mediated change in holding current elicited by CCh in cultured hippocampal neurons. In addition, since it was shown that mGluR5 levels are reduced at late stages of AD pathology but not at early stages, it was hypothesised that P-hTau expression does not reduce mGluR-mediated change in holding current elicited by DHPG in cultured hippocampal neurons. This study will help to understand the vulnerability of synaptic GPCRs to tau pathology and will provide valuable information regarding the role of tau in the disruption of synaptic function.

5.2 Results

5.2.1 CCh-induced holding current change is increased in P-hTau transfected neurons

To assesses the effects of phosphomimetic human tau (P-hTau) on mAChRs function, cultured hippocampal slices were transfected with P-hTau and rat tau shRNA (rTau-shRNA). This rTau-shRNA has been previously shown to successfully knock-down endogenous tau expressed in cultured hippocampal slices (Kimura et al. 2013; Regan et al. 2015). Analogously to field recording experiments (see **Chapter 3**), CCh was used to pharmacologically activate mAChRs. CCh produces a characteristic change in holding current during whole-cell recordings that can be used as a readout of mAChRs function (Fiszman et al. 1991; Haj-Dahmane & Andrade 1996; Hsu et al. 1996). This was corroborated in the present study as perfusion of CCh (50 µM, 5 minutes) resulted in a change in holding current with a peak of approximately 45 picoamperes (Untransfected: -44.86 ± 8.95, **Figure 5-1**). This change was significantly increased in P-hTau transfected neurons (P-hTau: -84.00 ± 11.11, p-value = 0.013, **Figure 5-1**). This data indicates that mAChRs activation in P-hTau transfected cells triggers a bigger holding current change, suggesting a tau-induced facilitation of mAChRs function.

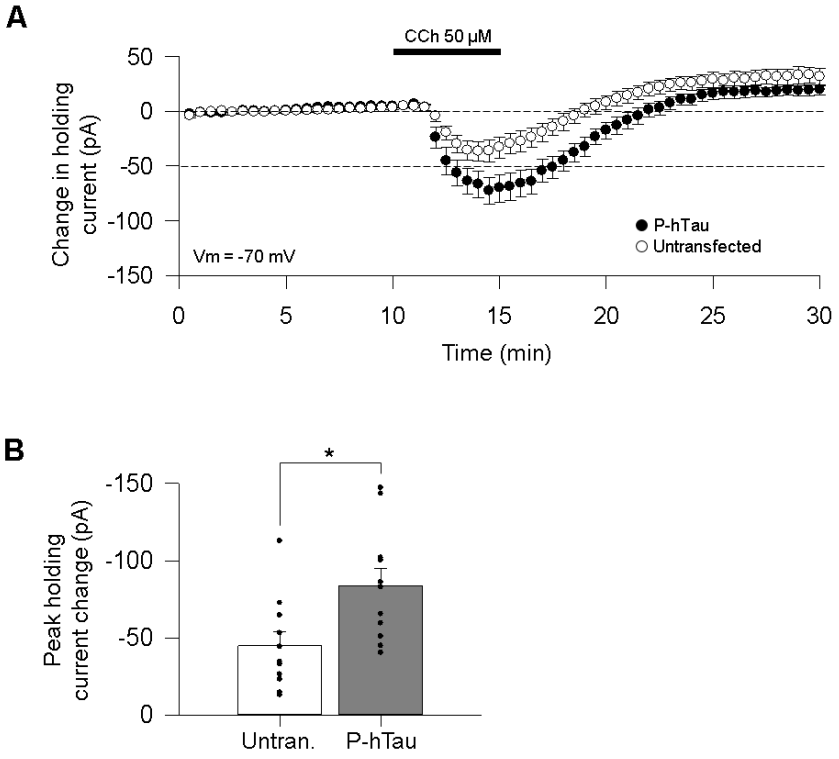


Figure 5-1. CCh-induced holding current change is increased in P-hTau transfected neurons. (A) Holding current change in untransfected (N = 11) and P-hTau transfected neurons (N = 11). **(B)** P-hTau transfected neurons have significantly higher peak in holding current change than untransfected neurons. Circles represent the mean and error bars the standard error of the mean (S.E.M.). Significance level (*$p < 0.05$) was determined by using an unpaired Student t-test.

5.2.2 CCh-induced holding current change is not altered in WT-hTau transfected neurons

The enhancement of CCh-induced holding current change could be due to the effect of tau but not specifically due to its phosphorylation state. To test this possibility, cultured hippocampal slices were transfected with wild-type human tau (WT-hTau) in addition to rTau-shRNA. WT-hTau construct encodes for full-length (2N4R) human tau but does not mimic phosphorylation. Perfusion of CCh resulted in a change in holding current in untransfected neurons that was not significantly altered in WT-hTau transfected neurons (untransfected: -63.90 ± 8.13, WT-hTau: 63.10 ± 6.76, p-value = 0.944, **Figure 5-2**). These data suggest that the enhancement of CCh-induced holding current change in P-hTau neurons is specifically due to the phosphorylation state of tau and not solely due to the presence of human tau.

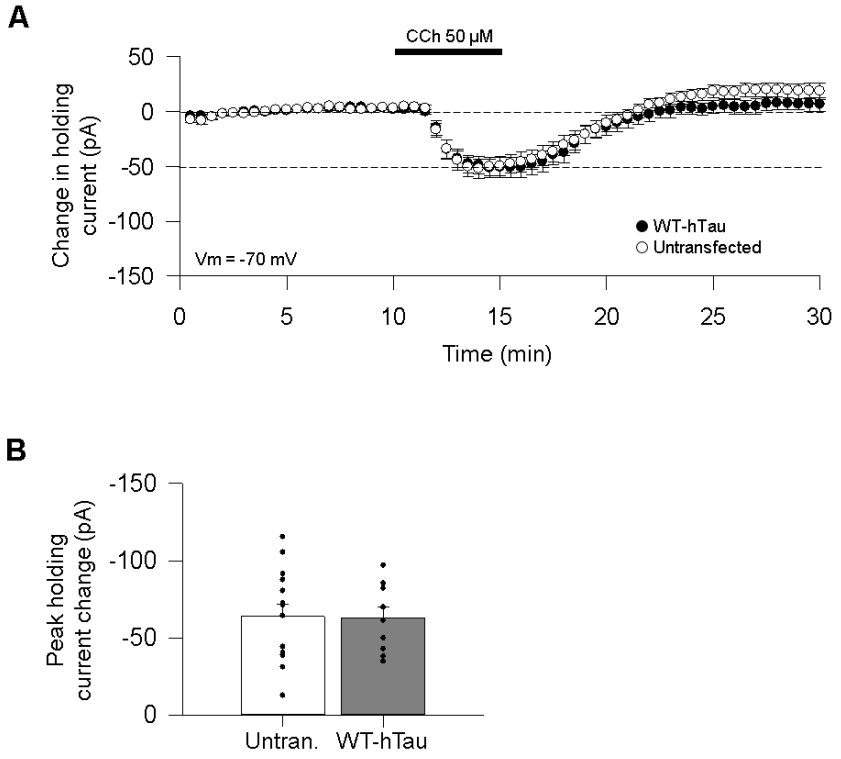


Figure 5-2. CCh-induced holding current change is not altered in WT-hTau transfected neurons. (A) Holding current change in untransfected (N = 14) and WT-hTau transfected neurons (N = 10). **(B)** No significant differences in peak holding current change between WT-hTau transfected neurons and untransfected neurons. Circles represent the mean and error bars the standard error of the mean (S.E.M.). Significance level (*p < 0.05) was determined by using an unpaired Student t-test.

5.2.3 CCh-induced holding current change is not altered in AT8E-hTau transfected neurons

Previous results showed that the CCh-induced holding current change was increased in P-hTau transfected neurons and that this effect was not due to the presence of tau itself but very likely due to its phosphorylation state. In the P-hTau construct, serine residues are mutated to glutamate so they mimic constitutive phosphorylation at three epitopes: AT8 (S199/S202/T205), AT100 (T212/S214), and PHF-1 (S396/S404). The term “epitope” is used for these sites as they are recognised by the antibodies AT8, AT100 and PHF1, respectively. Therefore, it was unknown whether the increase in CCh-induced holding current change involved phosphorylation at all epitopes or if there was a specific epitope responsible for this effect. To further clarify this, cultured hippocampal slices were transfected with AT8E-hTau construct in addition to rTau-shRNA. In these slices, CCh induced a holding current change in untrasfected neurons that was not altered in AT8E-hTau transfected neurons (Untransfected: -69.67 ± 5.50, AT8E-hTau: -

61.50 ± 11.67, p-value = 0.163, **Figure 5-3**). Therefore, this data suggests that the increase in CCh-induced holding current change observed in P-hTau transfected neurons is not due to phosphorylation of the AT8 epitope of human tau.

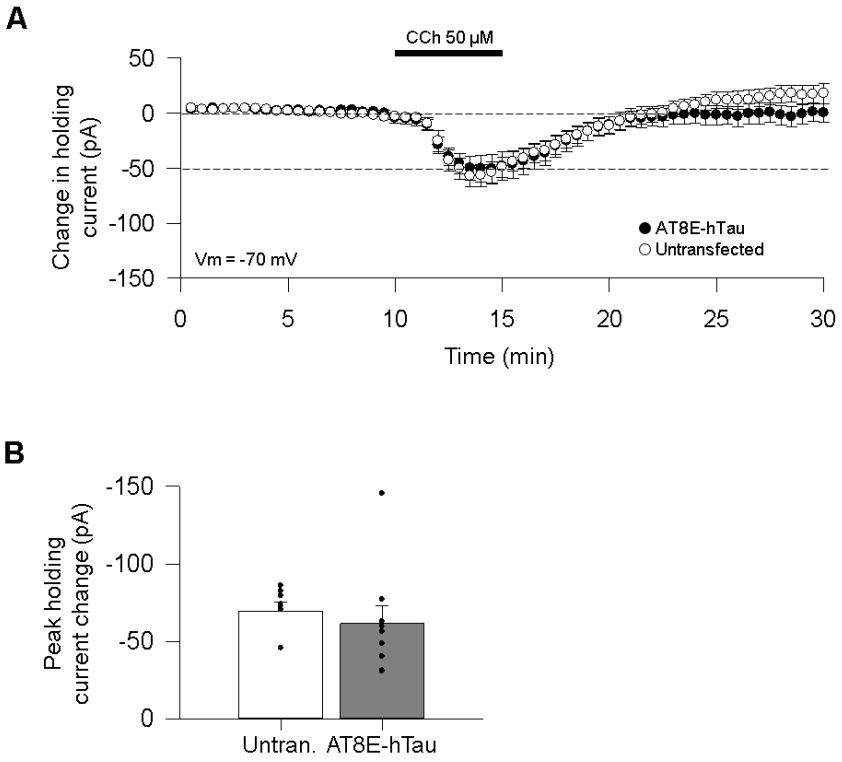


Figure 5-3. CCh-induced holding current change is not altered in AT8E-hTau transfected neurons. (A) Holding current change in untransfected (N = 8) and AT8E-hTau transfected neurons (N = 9). **(B)** No significant differences in peak holding current change between AT8E-hTau transfected neurons and untransfected neurons. Circles represent the mean and error bars the standard error of the mean (S.E.M.). Significance level (*p < 0.05) was determined by using Mann-Whitney rank sum test.

5.2.4 DHPG-induced holding current change is not altered in P-hTau transfected neurons

The aim of this study was to understand the effects of hyperphosphorylated tau on synaptic GPCR function. Since P-tau clearly affected mAChRs function, it was next aimed to test whether it could also affect the function of mGluRs, as they are synaptic GPCRs coupled to the same signalling pathway as mAChRs. Therefore, it was hypothesised that the effect of P-tau could be specific to a GPCR-mediated mechanism. To test this hypothesis, cultured hippocampal neurons were transfected with P-hTau and rTau-shRNA and perfused with the group I mGluRs agonist DHPG (20 µM, 5 min). These experiments showed that DHPG triggers a holding current change of similar magnitude to that elicited by CCh in untransfected

cells and that this effect was not altered in P-hTau transfected cells (Untransfected: -45.32 ± 7.11, P-hTau: -46.65 ± 5.20. p-value = 0.884, **Figure 5-4**). These results indicate that P-hTau does not affect group I mGluRs function as assessed by alteration of DHPG-induced holding current change.

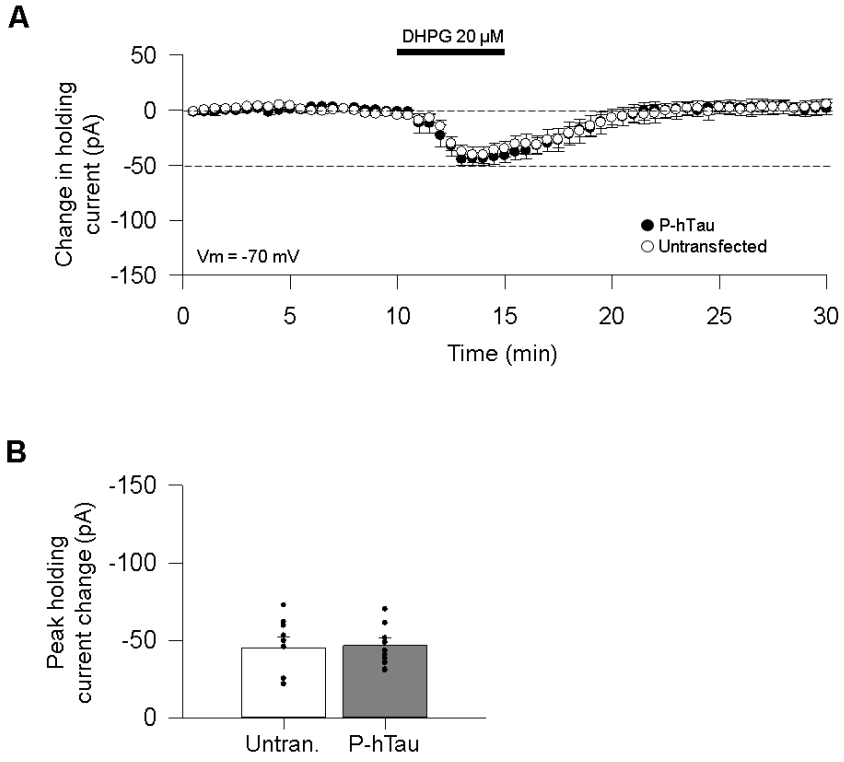


Figure 5-4. DHPG-induced holding current change is not altered in P-hTau transfected neurons. (A) Holding current change in untransfected (N = 6) and P-hTau transfected neurons (N = 6). **(B)** No significant differences in peak holding current change between P-hTau transfected neurons and untransfected neurons. Circles represent the mean and error bars the standard error of the mean (S.E.M.). Significance level (*p < 0.05) was determined by using an unpaired Student t-test.

5.2.5 DHPG-induced holding current change is not altered in WT-hTau transfected neurons

Although no effect was observed in DHPG-induced holding current change in P-hTau transfected neurons, it remained possible that tau itself could have an effect on the function of group I mGluRs. In support of this, full-length hTau is able to regulate GPCRs function (Gómez-Ramos et al. 2006; Gómez-Ramos et al. 2008; Gómez-Ramos et al. 2009). To test this possibility, cultured hippocampal slices were transfected with WT-hTau and rTau-shRNA and perfused with DHPG as in the previous experiment. This showed that DHPG-holding current change was not significantly different between untransfected neurons and WT-hTau transfected

neurons (Untransfected: -39.11 ± 5.20, WT-hTau: -48.37 ± 4.23, p-value = 0.125, **Figure 5-5**). In combination with the previous result, this data suggests that hTau, either constitutively phosphorylated or in its wild-type form, does not affect the function of group I mGluRs in the experimental conditions tested here.

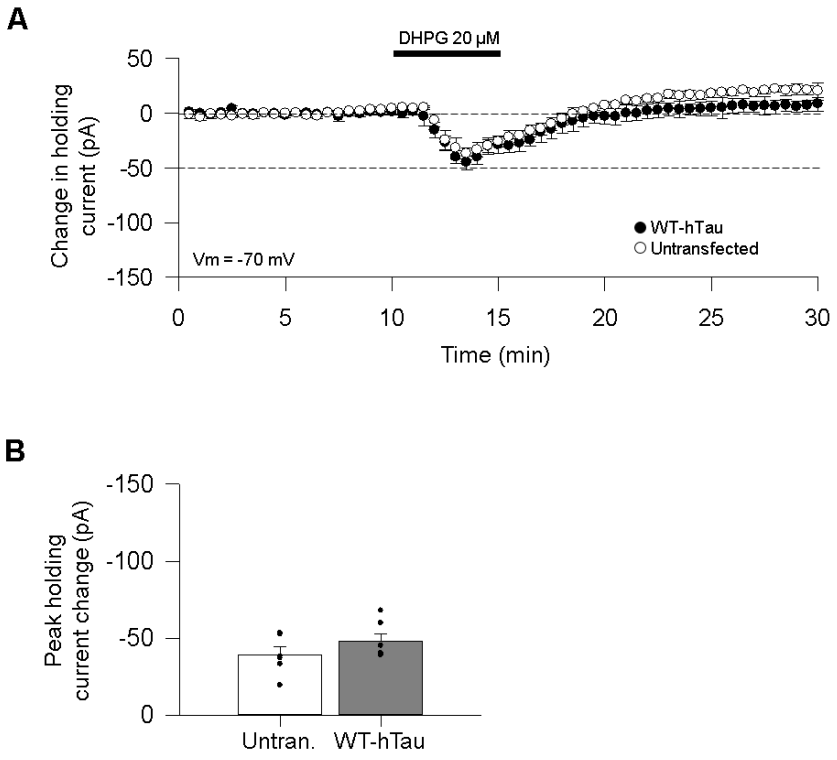


Figure 5-5. DHPG-induced holding current change is not altered in WT-hTau transfected neurons. (A) Holding current change in untransfected (N = 6) and WT-hTau transfected neurons (N = 7). **(B)** No significant differences in peak holding current change between WT-hTau transfected neurons and untransfected neurons. Circles represent the mean and error bars the standard error of the mean (S.E.M.). Significance level (*p < 0.05) was determined by using an unpaired Student t-test.

5.3 Discussion

A wide body of evidence supports the role of tau pathology as a potential cause of neurodegenerative diseases (Hutton et al. 1998; Nelson et al. 2012). However, the precise mechanisms by which it contributes to degeneration in these disorders are not fully understood. While NFTs are considered a neuropathological hallmark required for the *post-mortem* diagnosis of AD (Braak & Braak 1991), it is becoming clear that early synaptic dysfunction results from tau hyperphosphorylation (Ballatore et al. 2007). This aberrant post-translational modification of tau very likely results in synapse loss (Thies & Mandelkow 2007), defects in microtubule assembly and axonal transport (Alonso et al. 1994; Li et al. 2007; Alonso et al. 1996), and enhancement of Aβ-mediated toxicity (Roberson et al. 2007; Ittner et al. 2010; Rapoport et al. 2002; Shipton et al. 2011). In addition, tau-mediated disruption of function of receptors pivotal for memory, such as ionotropic glutamate receptors (Ittner et al. 2010; Mondragón-Rodríguez et al. 2012; Yu et al. 2012; Kopeikina et al. 2013), may provide a molecular basis for the cognitive decline associated with the degree of tauopathy (Polydoro et al. 2009; Fá et al. 2016; Nelson et al. 2012). However, the effects of P-Tau on other synaptic receptors also important for memory, such as metabotropic receptors, are much less understood.

As shown in **Chapter 4**, the molecular analysis of *post-mortem* brain samples revealed multiple alterations in the expression of synaptic receptors at different stages of AD progression. Such alterations may be driven by the presence of P-Tau in these brains and may have a functional impact on these receptors. Therefore, it was hypothesised that P-Tau could disrupt the function of synaptic GPCRs. In the case of mAChRs, CCh was perfused to the slices to induce a change in holding current measured in whole-cell recordings that was used as a readout of mAChRs function. In P-hTau transfected neurons, the CCh-induced holding current change was significantly increased (**Figure 5-1**) whereas no change was observed in WT-hTau transfected neurons (**Figure 5-2**). These results indicate the potential of P-hTau as a modulator of mAChRs function.

Interestingly, Tau-mediated modulation of cholinergic receptors has been previously reported (Simón et al. 2013). For example, intracellular accumulation of hTau can result in the calcium-dependent degradation of nicotinic receptors, causing a reduction in receptor expression (Yin et al. 2016). Another well-documented example is that extracellular hTau can interact with mAChRs,

particularly neuronal mAChR1 and mAChR3 receptors, to potentiate the intracellular calcium increase mediated by these receptors (Gómez-Ramos et al. 2006; Gómez-Ramos et al. 2008). In this study, the authors identified the C-terminal region comprising residues 391-407 of the full-length hTau as the one required for the interaction with mAChRs (Gómez-Ramos et al. 2008). Whether there is a direct interaction between P-hTau and mAChRs in the experimental model used here remains to be investigated. However, this may not to be the case as it was shown that tau phosphorylation prevents its interaction with mAChRs (Díaz-Hernández et al. 2010). Therefore, the mechanism that accounts for tau-mediated regulation of mAChRs in the present study may not require a direct interaction.

In the experimental conditions of the present study, the change in holding current observed by perfusion of CCh in cultured hippocampal slices is presumably due to an activation of mAChRs (Fiszman et al. 1991; Haj-Dahmane & Andrade 1996; Hsu et al. 1996). Receptor activation results in the depolarisation of the cell, an effect thought to be mediated by activation of voltage-dependent non-selective cation currents (Haj-Dahmane & Andrade 1996) in combination with inhibition of potassium currents (Hsu et al. 1996). As a consequence of membrane depolarisation, a higher current is applied to the cell in voltage clamp mode to keep it at the commanded voltage of -70 mV. If the presence of P-hTau results in a bigger holding current change, this could mean that P-hTau itself causes cell depolarisation, so the later activation of mAChRs by CCh occurs in an already depolarised state and the CCh-mediated effects on holding current add on to those of P-hTau. One potential way in which P-hTau may increase cell depolarisation is through indirect actions on NMDARs. As previously mentioned, it has been shown that hTau can induce the translocation of Fyn kinase to the dendritic compartment where it triggers Fyn-dependent phosphorylation of the NR2B subunit of NMDARs (Ittner et al. 2010). This facilitates the interaction of NMDARs with the PSD-95 (Rong et al. 2001) and couples the receptor to excitotoxic mechanisms (Ittner et al. 2010). NMDAR-mediated excitotoxicity involves the increase of intracellular calcium and cell depolarisation (Dong et al. 2009) that leads to cell death through activation of calpain (Vosler et al. 2008) and mitochondrial apoptotic pathways (Alberdi et al. 2010). Importantly, dendritic accumulation of hTau occurs in a phosphorylation dependent manner (Hoover et al. 2010). Therefore, it is possible that in the present study, the presence of P-hTau leads to an increased

depolarisation of neurons mediated by NMDARs, further increasing the CCh-induced depolarisation mediated by mAChRs.

To further investigate what phosphorylation sites are involved in the effect of P-hTau on CCh-induced holding current change, a phosphomimetic mutant for the AT8 site was used. Notably, neuropathological classification of human brain samples studied in **Chapter 4** was made according to AT8 immunostaining of the tissue, in accordance with the standardized Braak staging of NFTs distribution (Braak et al. 2006). In addition, phosphorylation at this epitope causes tau missorting to the dendritic region and subsequent neurotoxic effects (Zempel et al. 2010), leading to the idea that this epitope may be relevant for tau pathological effects. If the same change in holding current was observed in AT8E-hTau transfected cells than in P-hTau transfected cells, this would indicate that this site is involved in the regulation of mAChRs. This experiment showed that CCh-induced holding current change was not significantly different between untransfected and AT8E-transfected neurons (**Figure 5-3**). This data indicates that phosphorylation at the AT8 site is not required for the regulation of mAChRs function by P-hTau but does not discount the involvement of other sites. The hyperphosphorylated mutant form of tau used in this study (P-hTau) corresponds to the full-length hTau containing mutations in amino acid residues that mimic constitutive phosphorylation at three different sites: AT8, AT100 and PHF1. Therefore, the possibility that AT100 and/or PHF1 are involved in the CCh-mediated change in holding current cannot be excluded. This would not be surprising since phosphorylation tightly regulates tau function and specific effects of the protein are mediated by phosphorylation at specific sites (Augustinack et al. 2002).

Although phosphorylation is considered the major post-translational modification of tau, other post-translational modifications play a role in its pathological effects (Martin et al. 2011). These include glycosylation, nitration, truncation, polyamination and ubiquitination, among others. Likely, interactions between several post-translational modifications take place to drive tauopathy. For example, glycosylation may induce conformational changes in tau structure that expose phosphorylation sites, partially explaining why glycosylation precedes hyperphosphorylation (Liu et al. 2002; Yuzwa et al. 2008). In the experimental model presented here, tau mutants already include the residues substitutions that

mimic phosphorylation. Therefore, this limits the physiological relevance of these mutants as some post-translational modifications that would normally occur after the protein is transcribed may not happen due to steric hindrance by modified residues. In addition, the temporal sequence in which post-translational modifications occur may also have pathological implications. Illustrating this, sequential phosphorylation of tau at specific residues generates the AT100 epitope and this affects the microtubule-binding capacity of tau (Yoshida & Goedert 2006). According to this evidence, it cannot be excluded that other post-translational modifications occur in this experimental model and therefore contribute to the effect of P-hTau on mAChRs function.

Results from this study support that P-hTau differentially regulates the function of mAChRs and mGluRs. Since experiments were carried out 3 days after biolistic transfection of tau mutants into cultured hippocampal slices, they are more likely to reflect early effects of P-hTau expression. This would mean that P-hTau alters the function of mAChRs in the first place but other receptors resist this insult. This is potentially consistent with data from **Chapter 4** showing a reduced expression of mAChRs at earlier stages of AD pathology and mGluRs being reduced at late stages. This may mean that mGluRs are more resilient to the effects of tau pathology and consequently become affected when P-hTau has been present for longer periods of time in the brain.

The differential regulation of mGluRs and mAChRs by P-hTau rises yet again the question of what the reasons for this difference may be, given that both GPCRs couple to the $G_{q/11}$ pathway. As discussed in **Chapter 3**, different functional effects of activation of GPCRs coupled to the same pathway may relay on differential location (Moore et al. 2009; Ostrom & Insel 2004) or different signalling cascades being activated in response to the same G-protein mechanism (Berkeley et al. 2001). According to data presented here, it is only possible to speculate that P-hTau firstly affects mAChRs, consistent with early impairments in cholinergic function in the presence of AD pathology. It remains to be elucidated why mAChRs are particularly vulnerable to early signs of pathophysiological changes and why mGluRs may be spared at this stage (see **Chapter 6**).

Chapter 6 General discussion

6.1 Summary of results

6.2 mGluR5 is not an isolated player in AD pathology

6.3 mGluR-mediated inhibition of mAChRs

6.4 Differential effects of AD pathology on synaptic GPCRs

6.5 Relevance of tau isoforms

6.6 Conclusions

6.1 Summary of results

The present thesis has provided the following findings:

1. Activation of mGluRs does not affect baseline recording or LTP induction.

2. Activation of mGluRs does not affect mAChRs-induced depression of fEPSPs triggered by CCh.

3. Activation of mGluRs reduces mAChRs-induced calcium increase triggered by CCh.

4. Activation of mGluRs does not affect the activation of GSK3β.

5. Protein expression of mAChRs and GluA2 is reduced in P-AD brains whereas mGluR5, NR1 and Homer 1b/c are reduced in AD brains.

6. Expression of P-hTau enhances mAChRs-induced holding current change triggered by CCh but not by DHPG.

6.2 mGluR5 is not an isolated player in AD pathology

Compelling evidence supports that synapses are the initial target of pathophysiology in AD (Selkoe 2002). Although this targeting eventually leads to synapse loss and subsequent loss of whole neurons, evidence points to deficiencies in synaptic function occurring first (Selkoe 2002; Pooler et al. 2014; Tu et al. 2014). Weakening of synaptic function is mediated by several signalling cascades that normally operate in physiological conditions but are hijacked in the presence of pathological insults. As outlined in **Chapter 3**, this thesis has provided support for this concept by showing that in non-disease conditions, the activation of mGluR5, a mediator of Aβ-induced neurotoxicity, does not disrupt synaptic plasticity. As discussed before, this is relevant to AD because it indicates that mGluR5 activation is not sufficient to cause synaptotoxicity. Instead, other molecules and pathways that are altered in pathology likely contribute to make mGluR5 into a key pathological mediator of Aβ-induced synaptic deficits. The question that remains to be answered is therefore: what are the

molecules/pathways activated by Aβ that may contribute to mGluR5 aberrant function?

To evaluate possible answers to such a challenging question, it is necessary to first consider the effects of Aβ on synaptic function. These include the blockade of LTP (Walsh et al. 2002; Cleary et al. 2005; Jo et al. 2011) and enhancement of LTD (J. H. Kim et al. 2001; Li et al. 2009; Hsieh et al. 2006), among other effects. Importantly, these deficits in synaptic plasticity have been linked to cognitive impairments observed in behavioural tests (Walsh et al. 2002; Selkoe 2008) and occur concomitantly to reductions in spine numbers (Wei et al. 2010). These findings indicate that Aβ clearly targets synapses (Lacor et al. 2004) and highlight the relevance of Aβ-induced synaptic deficits in the pathology of AD. Obviously, for Aβ to induce such effects, somehow its interference with intracellular cascades that modulate synaptic plasticity and/or function would be required. One compelling mechanism by which this is very likely to occur is the binding of Aβ to synaptic plasma membranes through its interaction with synaptic receptors (Dinamarca et al. 2012). In this way, Aβ can interfere with receptor activation and downstream signalling which in turn are necessary for the operation of synaptic plasticity mechanisms. Various Aβ receptors have been proposed, and interestingly some of them are as well able to regulate mGluR5 signalling.

For instance, different NMDAR subunits were co-immunoprecipitated with Aβo and an antibody against the NR1 subunit abolished neuronal binding of Aβo (De Felice et al. 2007). Another NMDAR subunit, NR2B, was co-localised with Aβo in hippocampal slices (Deshpande et al. 2009). Although the domain responsible for the interaction has not been characterised yet, it has been shown that Aβ binds in a NR1- and NR2B-dependent manner to hippocampal neurons (Lacor et al. 2004; Lacor et al. 2007). The functional relevance of this binding may be related to the Aβ-mediated LTP impairment that occurs in combination with activation of MAPK and downregulation of CREB signalling in a NR2B-dependent manner (Li et al. 2011). In addition, a functional regulatory interplay between NMDARs and mGluRs may play a role in Aβ pathology. It has long been reported that activation of mGluR5 can enhance NMDAR responses *in vitro* (Fitzjohn et al. 1996; Awad et al. 2000; Doherty et al. 1997; O'Connor et al. 1994; Attucci et al. 2001). In an animal model, mGluR5 antagonism potentiates the impairments in learning and memory in behavioural tasks caused by NMDAR blockade (Homayoun et al. 2004),

indicating that mGluR5 regulation of NMDARs function may be relevant for NMDAR-dependent cognitive tasks.

Importantly, this regulatory effect is not unidirectional, as NMDARs can also inhibit or potentiate mGluRs-mediated signalling and particularly that of mGluR5, depending on the NMDA concentration (Alagarsamy et al. 2002; Luthi, et al. 1994; Challiss et al. 1994). Illustrating this, NMDAR activation results in dephosphorylation of mGluR5, reversing its PKC-dependent desensitisation and resulting in the potentiation of mGluR5-mediated inward currents recorded in acute hippocampal slices (Alagarsamy et al. 1999). Furthermore, NMDA treatment of cortex slices results in the potentiation of mGluR-induced phosphoinositide hydrolysis (Challiss et al. 1994). As mentioned before, this functional interaction between NMDARs and mGluR5 has the potential to be relevant for AD mechanisms since pathological activation of NMDAR by Aβ has been well-established (Molnár et al. 2004; Miguel-Hidalgo et al. 2002; Texidó et al. 2011; Li et al. 2011). This, together with findings showing the potential binding of Aβ to NMDARs and that NMDARs can modulate mGluR5 function, provides a solid basis for the idea that the widely reported aberrant activity of mGluR5 in AD-like pathological conditions could be partially due to the regulatory effect of Aβ-induced NMDAR overactivity on mGluR5. Therefore, it is plausible that the lack of overactive NMDARs and associated signalling spares activation of mGluR5 as an innocuous event for synaptic function in the physiological model used in the present study.

In addition to reducing phosphorylation-dependent mGluR5 desensitisation, another potential mechanism by which NMDARs may cause the potentiation of mGluR5 signalling is an increase in intracellular calcium levels. As mentioned before, a well-stablished theory is that Aβ causes aberrant activation of NMDARs (Molnár et al. 2004; Miguel-Hidalgo et al. 2002; Texidó et al. 2011; Li et al. 2011). This is further supported by the fact that an FDA-approved treatment for AD is memantine, an NMDAR blocker (Alzheimer's Association 2018). Pathological activation of NMDARs is thought to result in an increased amount of calcium influx entering the cell through the ion channel which triggers a variety of synaptotoxic mechanisms (Kelly & Ferreira 2006; Mattson et al. 1992; Dong et al. 2009; Harkany et al. 2000). This is not surprising considering that calcium is a crucial second messenger that regulates a vast amount of key neuronal processes, including

neuronal growth (Henley & Poo 2004), neurotransmitter release (Südhof 2012a), synaptic plasticity (Fitzjohn & Collingridge 2002) (Fitzjohn 2002) and cell death (Zhivotovsky & Orrenius 2011). As a result, dysregulation of calcium homeostasis is the gateway for severe disruptions in neuronal function and structure that characterise neurodegeneration (Wang et al. 2017). Supporting this, incubation of cortical cultures with Aβ peptides results in release of calcium from the endoplasmic reticulum (ER), which is required for Aβ-induced neuronal death (Ferreiro et al. 2004). In addition, ER calcium release dependent on ryanodine receptor is markedly increased in hippocampal slices from an AD mouse model, and it is required for the disruption of synaptic transmission (Chakroborty et al. 2009). *In vivo*, Aβ-induced aberrant calcium increase results in loss of spino-dendritic calcium compartmentalisation and dystrophy of neurites (Kuchibhotla et al. 2008). This evidence has provided the foundations of the compelling theory that establishes calcium excitotoxicity as a major feature of AD-like pathology (Demuro et al. 2010).

Interestingly, by a different molecular mechanism than NMDARs, activation of mGluR5 also leads to an increase in intracellular calcium levels. Presumably, in conditions where this activation is excessive, such as in the presence of Aβ (Kumar et al. 2015; Wang 2004; Hu et al. 2014; Um et al. 2013; Hamilton et al. 2014; Haas et al. 2016), this effect is exacerbated. Although there is no direct evidence supporting that the detrimental effects of mGluR5 activation on synaptic function involve mGluR5-activated calcium-dependent pathways, this is quite likely given that this is a prominent signalling pathway triggered by $G_{q/11}$-coupled receptors, such as mGluR5 (Huang & Thathiah 2015). Therefore, one may think that in physiological conditions, agonist activation of mGluR5 results in a calcium increase that is not high enough to trigger neurotoxicity. However, if this occurs in combination with NMDAR activation and/or Aβ-induced calcium alterations, intracellular calcium concentration may reach a “pathological level” over which toxic signalling could be engaged and pathology triggered. Indeed, it is known that cells maintain cytosolic calcium levels within the nanomolar range (Gleichmann & Mattson 2011) and that the changes induced by Aβ greatly exceed the resting calcium levels (Kuchibhotla et al. 2008). Therefore, in addition to the previously mentioned synergistic effect of NMDARs and mGluRs, summation of their calcium signalling may be a key intracellular change in making mGluR5 a pathological mediator.

Another Aβ receptor that is in turn a promising candidate for turning mGluR5 into a key mediator of Aβ-synaptotoxicity is cellular prion protein (PrP^C). Support for this relies on the demonstration that Aβo bound to PrP^C interact with mGluR5 (Hamilton et al. 2015; Um et al. 2013; Haas et al. 2016). This may correlate with the increased vulnerability of mGluR5 bearing neurons to Aβ pathology (Overk et al. 2014; Beraldo et al. 2016). Notably, the indirect interaction of Aβ with mGluR5 can result in the overstabilization of mGluR5 at the postsynaptic membrane and subsequent aberrant activation of the receptor (Renner et al. 2010). This may explain why the reduction of mGluR5 activity by pharmacological (Hamilton et al. 2016; Um et al. 2013) and genetic approaches (Hamilton et al. 2014) reverses Aβ-induced deficits in learning and memory, as well as it ameliorates Aβ-mediated calcium toxicity (Renner et al. 2010; Um et al. 2013) and spine density (Um et al. 2013). In addition, this interaction may provide a mechanistic basis for the involvement of mGluR5 in the *in vivo* Aβ-mediated facilitation of LTD, for which both PrP^C and mGluR5 are required (Hu et al. 2014). This evidence suggests that Aβ binds to PrP^C and this complex interferes with mGluR5 physiological signalling (Renner et al. 2010).

For instance, aberrant activation of mGluR5 within the PrP^C-mGluR5 complex results in the cytoplasmic activation of the kinase Fyn (Um et al. 2012; Um et al. 2013; Larson et al. 2012). Fyn then phosphorylates tau and NMDARs in an Aβ-induced manner, inducing excitotoxicity and spine loss (Um et al. 2012; Larson et al. 2012). This role of mGluR5 as a central transducer of Aβ-mediated effects on synaptic function, may explain why its blockade with antagonists may be sufficient to prevent pathological signalling from the complex when Aβ is present (Haas & Strittmatter 2016; Haas et al. 2014). Furthermore, a recent study has shown that specific inhibition of pathological Aβ-mediated signalling through mGluR5 can restore behavioural and memory deficits, synaptic depletion and tau pathology in an AD transgenic mice model (Haas et al. 2017). They use a specific mGluR5 silent allosteric modulator (BMS-984923) (Huang et al. 2016) that blocks pathological Aβ-induced signalling and enhancement of PrP^C-mGluR5 interaction without altering basal glutamate-induced calcium signalling (Gregory et al. 2010; Gregory et al. 2011; Sheffler et al. 2011). These findings further support that there is a clear separation between pathological and physiological molecular roles of mGluR5 (Haas et al. 2017). With these ideas on scope, mGluR5 activation may not be

sufficient to cause synaptic impairments as those mediated by the receptor in disease-like conditions due to the absence of synergistic activation by PrP^C.

The fact that mGluR5 is not the only key for AD pathology has therapeutic implications. Importantly, drugs targeting mGluR5 may not be a successful intervention unless used in combination with others aimed to tackle aspects of synaptic pathology occurring simultaneously to mGluR5 activation. Indeed, the need of using combined therapies that tackle more than one neuropathological problem has become a reality now (Hendrix et al. 2016; Jarosz-Griffiths et al. 2016).

6.3 mGluR-mediated inhibition of mAChRs

GPCRs expressed on the postsynaptic membrane play important roles in the regulation of synaptic function (Betke et al. 2012). As outlined extensively throughout this thesis, efficient synaptic transmission and the integrity of synaptic connections are essential for correct functioning of neuronal communication (Huang & Thathiah 2015). Consequently, disruption of GPCRs expression or function is a common feature in numerous brain disorders, including AD (Huang et al. 2017). As previously discussed, reduction of cholinergic transmission is a hallmark of AD pathology (Whitehouse et al. 1982; Davies & Maloney 1976; Parent et al. 2013) and functional downregulation of the synaptic GPCRs mAChR has been proposed as a contributing factor to molecular and cognitive deficits in AD models (Caccamo et al. 2006; Davis et al. 2010). The outstanding question is however what the mediators for mAChRs dysregulation are. In this regard, data presented in **Chapter 3** provides evidence for a possible inhibitory effect of mAChRs function exerted by group I mGluRs. Considering the variety of effects mediated by mAChR and mGluRs in addition to the significant number of intracellular molecules in common, some possible molecular mechanisms for this interaction may be considered.

Regulation of GCPRs activity is an orchestrated process that occurs at many functional levels and involves a wide variety of GPCR-interacting proteins (Magalhaes et al. 2012). These include GPCR kinases (GRKs) (Ribas et al. 2007), regulators of G-protein signalling (RGS) (Nunn et al. 2006; Stewart & Fisher 2015), β-arrestins (Smith & Rajagopal 2016) and receptor activity-modifying proteins (RAMPS) (Hay & Pioszak 2016), among many others. In addition, GPCR-cross

talk mechanisms have been widely regarded as a way to control GPCRs function (Cordeaux & Hill 2002). This is not surprising considering that the number of GPCR effectors is smaller than that of receptors and this number is limited within the cell (Hur & Kim 2002). Therefore, one possibility is that cross-regulation between GPCRs occurs at points of the signalling pathway where common effectors are required (see **Chapter 3**, **section 3.3.2**). Alternatively, cross-regulation may occur as a result of one GPCR being able to regulate the above mentioned GPCR-interacting proteins that in turn regulate another GPCR.

Among the GPCR-interacting proteins that regulate mAChRs function, RGS proteins seem to have an important role. For instance, RGS2 can selectively bind to the third intracellular loop of mAChR1 and potently inhibit mAChR-mediated phosphoinositide hydrolysis (Bernstein et al. 2004). This is in agreement with the canonical mode of action of RGS proteins, consisting in inactivation of G_{α} subunits by accelerating the hydrolysis of GTP through stimulation of GTPase activity (Stewart & Fisher 2015). Although the regulation of RGS proteins is still poorly understood, it has been reported that PIP_3 can inhibit the GTPase-activating function of RGS in a calcium/calmodulin dependent fashion (Popov et al. 2000). The physiological relevance of this regulatory mechanism has been tested in cardiac myocytes, where PIP_3-dependent inhibition of RGS4 was reversed by calcium/calmodulin activation, resulting in decreased potassium channel activity induced by the GTPase activity of RGS4 (Ishii et al. 2002). Notably, this mechanism is regulated by elevation of intracellular calcium, which then binds to calmodulin to activate it (Ishii et al. 2002). Interestingly, calcium release from intracellular stores represents an intersection for signalling pathways triggered by $G_{q/11}$ GPCRs, including mGluRs (Werry et al. 2003). According to this, agonist activation of mGluRs resulting in the release of calcium from intracellular stores could cause the activation of RGS proteins that in turn regulate muscarinic actions.

In addition to RGS-mediated mechanisms, mAChRs activity is regulated by phosphorylation events at serine and threonine residues located on the receptor extracellular loops (Haga et al. 1990; Kwatra & Hosey 1986). These can be phosphorylated by numerous kinases among which are casein kinase 1a (CK1a), GRKs and PKC (van Koppen & Kaiser 2003; Haga et al. 1996; Tsuga et al. 1998; Budd et al. 2000). Importantly, GRK2 and GRK3 have been shown to supress $G_{q/11}$ signalling (Carman et al. 1999; Ribeiro et al. 2009; Willets et al. 2001). A more

relevant aspect for brain function is that GRK2 can inhibit mAChR1-induced IP_3 formation irrespectively of its kinase activity in cultured hippocampal neurons (Willets 2004). This study also showed that GRK2 is expressed in hippocampal neurons, suggesting its role in the regulation of hippocampal GPCRs (Willets 2004). This finding implies that GRK2 is endogenously expressed in the hippocampus and that it can regulate mAChR1 function in a phosphorylation independent manner in the hippocampus, which is in line with other study (Willets et al. 2003). Since the demonstration that GRK2 contains a sequence that can bind to $G_{q/11}$ (Sterne-Marr et al. 2003), it has been proposed that a direct interaction mediates its phosphorylation-independent effects on mAChR1 (Day et al. 2004; Lodowski et al. 2003). This does not exclude the regulation of mAChR1 by phosphorylation-dependent mechanisms, as this has also been demonstrated to occur in native neuronal conditions (Willets et al. 2005). Interestingly, GRK2 is in turn regulated by phosphorylation events (Chuang et al. 1995; Winstel et al. 1996). Particularly, PKC phosphorylation at the N-terminus of GRK2 prevents its inhibition by calmodulin (Krasel et al. 2000; Levay et al. 1998). According to this evidence, PKC activation may exert regulatory effects on mAChRs function through the activation of GRK2. It is well-established that mGluR5 activation results in PKC activation via the G-protein activated PLC/DAG pathway (Ferraguti & Shigemoto 2006). Therefore, this could provide another mechanism by which mGluR5 can induce the inhibition of mAChRs function.

In summary, regulators of GPCR activity such as RGS and GRKs may provide a molecular substrate for the mGluRs-mediated inhibition of mAChRs activity. Although their importance in the regulation of synaptic GPCRs cross-talk is yet to be explored, there are reasons to think that they may play such a role and consequently may be considered as targets to regulate GPCRs interplay.

6.4 Differential effects of AD pathology on synaptic GPCRs

Although multiple neurotransmitter systems are altered over the course of AD, dysfunction of glutamatergic and cholinergic synapses is better correlated with early symptomatology (Selkoe 2002). This is supported by findings shown in **Chapter 4**, where a selective loss of mAChR1 expression was observed in the brains of patients at earlier stages of AD pathology (Braak stages III-IV) but not at later stages (Braak stages V-VI). This specific downregulation of receptor expression in P-AD brains may reflect a very selective targeting by pathological

molecules present in this tissue, such as P-hTau. This is consistent with data provided in **Chapter 5**, showing that P-hTau, a key entity in AD brains used for their neuropathological evaluation, can regulate the function of mAChRs but not that of other synaptic GCPRs such as group I mGluRs. In the particular experimental conditions of this study, P-hTau was shown to upregulate the responses of mAChRs in conditions of receptor activation. Although the mechanism for this effect remains to be elucidated, it is possible to consider potential ways in which P-hTau may regulate mAChRs based on the reported effects of tau on other receptors expressed at the synapse.

The regulation of synaptic receptor function by tau has been widely studied, especially in the case of ionotropic receptors, AMPARs and NMDARs. As an illustrative example, tau enhances the interaction between GluA2 subunit of AMPARs and PICK1 (Yagishita et al. 2015). The functional relevance of this has been related to the involvement of these proteins in AMPAR trafficking and LTD mechanisms (Terashima et al. 2008; Hanley 2008; Steinberg et al. 2006; C.-H. Kim et al. 2001; Citri et al. 2010). Further supporting this, AMPARs surface expression is reduced in an animal model of tauopathy (Kopeikina et al. 2013) and in neuronal cultures where tau is present (Hoover et al. 2010; Yu et al. 2012). Given the importance of AMPARs for synaptic plasticity (Henley & Wilkinson 2016), these effects of tau on AMPAR trafficking have been proposed as a mechanism by which tau disrupts synaptic plasticity (Polydoro et al. 2009; Fá et al. 2016). Further supporting the ability of tau to regulate synaptic receptors function, tau-mediated regulation of NMDARs has been reported. As mentioned before in this thesis, a well-characterised example is that full-length hTau can induce excitotoxic activity of NMDARs via promoting Fyn trafficking to the synaptic compartment (Ittner et al. 2010). This effect is generated by Fyn phosphorylation of the NR2B subunit of NMDAR, resulting in enhancement of stability of the receptor at the PSD (Ittner et al. 2010; Mondragón-Rodríguez et al. 2012). In line with these studies, tau can enhance NMDARs activity with pathological consequences as full-length hTau resulted in NMDAR-toxicity in neuronal cultures in a NR2B-dependent manner (Amadoro et al. 2006). In addition, expression of tau in mouse hippocampal neurons resulted in increased NMDAR-induced excitotoxicity, dependent on the Fyn-mediated phosphorylation of tau (Miyamoto et al. 2017). Although the mechanisms for these regulatory effects are not clear yet, this evidence supports

that tau can alter the activity of NMDAR in a way that potentiates receptor-mediated excitotoxicity, probably through phosphorylation.

As previously mentioned for mGluR5, there is evidence supporting a regulatory interplay between NMDARs and GPCRs (Lu et al. 1999), suggesting that tau-mediated activation of NMDARs may impact GPCR function. For instance, activation of mAChRs can enhance NMDAR-mediated neurotransmission (i.e. EPSPs) in the auditory cortex, an effect dependent on G-protein activation (Aramakis et al. 1997). Furthermore, CCh administration in cortical slices resulted in an increase in NMDAR-evoked adenosine release (Semba & White 1997). Such interaction seems to also take place in the hippocampus, as ACh administration in hippocampal neurons was able to facilitate the slow component of NMDAR-EPSPs generated by NMDAR activation (Markram & Segal 1990) and CCh treatment of hippocampal slices transiently enhanced NMDAR-mediated inward currents (Auerbach & Segal 1996). Although these studies provide evidence for enhancing effects of muscarinic regulation on NMDARs activity, this functional interaction can be of inhibitory nature. Illustrating this, mAChRs activation can result in downregulation of NMDAR-mediated inward currents in CA3 neurons through a mechanism involving G-protein mediated calcium release (Grishin et al. 2005). This heterogeneity of effects indicates the complexity of cross-talk between cholinergic and glutamatergic systems. Further supporting this concept, NMDAR can in turn downregulate the phosphoinositide response induced by mAChRs activation in cerebellar granule neurons (Butcher et al. 2009). In this study, the authors showed that activation of NMDARs results in the activation on calcium/calmodulin-dependent kinase II (CaMKII) and proposed that CaMKII can then phosphorylate mAChR causing its uncoupling from $G_{q/11}$ and subsequent desensitisation (Butcher et al. 2009). Moreover, antagonism of NMDARs enhanced the detrimental effects of mAChRs blockade in behavioural tasks (Moreira et al. 2005), further supporting the functional relevance of this regulatory interaction in the brain. Altogether this evidence supports that NMDAR can regulate mAChR G-protein coupled actions.

According to the above, since hTau can induce aberrant activation of NMDARs and associated neurotoxicity and NMDARs can result in the enhancement of mAChRs activity, a potential mechanism underlying the P-hTau-mediated potentiation of mAChRs function reported in the present study is the overactivation

of NMDARs by P-hTau. This possibility remains to be determined but it could be relevant for AD pathological mechanisms, as overactivation of NMDARs is a feature of pathology (Molnár et al. 2004; Miguel-Hidalgo et al. 2002; Texidó et al. 2011; Li et al. 2011).

The fact that the enhancing effect of P-hTau on mAChR activation was not observed for group I mGluRs indicates that for some unidentified reason, mGluRs are more resistant to the presence of pathological tau. If assuming the hypothesis that NMDARs activation is a mechanism by which enhancement of mAChRs-induced holding current change occurs, the lack of effect of P-hTau on mGluRs activation may rely on a specific feature of the modulatory effect of NMDARs on mGluRs.

Interestingly, NMDAR-mediated enhancement of mGluRs involves the activation of a phosphatase. This is supported by studies showing that the potentiation of mGluR5-mediated phosphoinositide hydrolysis in rat cortex depends on the NMDARs-dependent activation of calcineurin (Alagarsamy et al. 1999; Alagarsamy et al. 2005). According to these studies, activation of calcineurin results in the dephosphorylation of mGluR5 at serine/threonine residues located at the C-terminal domain, impeding its PKC-mediated desensitisation. Notably, the authors also provide evidence that the calcineurin mediated effect is due to the association of calcineurin with mGluR5 within a signalling complex in the PSD. The authors proposed that a possible intermediary between calcineurin and mGluR5 may be calmodulin, given that is both necessary for calcineurin activation (Rumi-Masante et al. 2012) and it can bind to the C-terminal domain of mGluR5 (Minakami et al. 1997). Interestingly, calcineurin has been widely studied in the context of AD due to its ability to dephosphorylate tau (Reese & Taglialatela 2011). Indeed, in the presence of tau, calcineurin binds to the calmodulin-binding domain of tau to exert dephosphorylation effects in mouse brain extracts (Yu et al. 2008). According to this evidence, it is possible that in the experimental conditions of the present study, the intracellular expression of P-hTau sequesters calcineurin proteins present in the cytoplasmic space. In this case, calcineurin would no longer be available to mediate NMDARs-induced potentiation of mGluRs-mediated effects (Alagarsamy et al. 2005) triggered by P-hTau. Importantly, this would not necessary affect NMDAR-induced potentiation of mAChRs-mediated holding current change. Testing this hypothesis will require future work which would indeed help to

understand the differential sensitivity of glutamatergic and cholinergic systems to acute effects of P-hTau.

Reconcile the up-regulation of mAChR-mediated responses by P-hTau with the downregulation of receptor expression in neurodegenerative brains seen in **Chapter 5** is challenging considering the intrinsic limitations of comparing an *in vitro* model with *post-mortem* human tissue preparations. However, leaving these limitations aside, one may consider that if P-hTau triggers an upregulation of mAChRs function this would lead to desensitisation of the receptor in the long-term. It is important to note that in the present study, electrophysiology experiments may reflect effects of acute P-hTau expression, as they were performed 3-5 days after transfection of tau constructs. Although this may serve as an approximation to changes occurring at initial phases of AD, intracellular accumulation of P-hTau persists over time in the human diseased brain (Braak & Braak 1991). As a result, chronic enhancement of mAChRs function possibly leads to the activation of receptor downregulation mechanisms to counterbalance the initial exacerbated function (van Koppen & Kaiser 2003). Indeed, this is a common regulatory mechanism of GPCRs function (Grady et al. 1997; Kelly et al. 2008) that applies to agonist-activation of mAChRs (Lee & El-Fakahany 1987; Li et al. 2003). In addition, it has been shown that agonist-activation of mAChRs results in receptor loss due to increased degradation (Roskoski et al. 1985), explaining an overall loss of mAChRs expression. However, this would not explain why at later stages of AD pathology, the levels of mAChR in the brains of AD patients remain unchanged when compared to control subjects. Considering that the effects of pathology on mGluRs function were different from those on mAChRs, i.e. downregulation of mGluR5 expression was observed at late stages and that P-hTau did not affect mGluRs function; a possible mechanism that conciliates these findings may emerge from the interplay between mGluRs and mAChRs reported in this study.

First, although the analysis of protein expression in AD brains is performed at a concrete time point, the outcome of this analysis is likely to reflect biochemical changes to which these brains have been subjected to for a long period of time. As outlined before in this thesis, one of these biochemical changes is probably the accumulation of glutamate. This is substantiated by *in vitro* findings showing that Aβ can induce reduced glutamate clearance (Li et al. 2009; Parpura-Gill et al. 1997; Fernández-Tomé et al. 2004) and potentiate glutamate release (Kabogo et

al. 2010; Arias et al. 1995; Abramov et al. 2009). In addition, a strong support emerges from studies of human AD brains showing reductions in glutamate transporters (Lauderback et al. 2001; Li et al. 1997; Jacob et al. 2007; Scott et al. 2011; Kashani et al. 2008; Kirvell et al. 2006). Therefore, a prevalent hypothesis is that glutamate accumulates throughout AD progression and in doing so causes excitotoxic effects (Hynd et al. 2004a). Hence, these effects may be gradual as glutamate builds-up (Butterfield & Pocernich 2003). Importantly, the temporal occurrence of pathological changes to the glutamatergic system may be of crucial importance for therapeutic strategies aimed to restore disruptions of this system in AD. Supporting this concept, a two-stage model of neurodegeneration was proposed by Olney and colleagues (1997). In this model, they stated that accumulation of Aβ results in hyperactivity of NMDARs at the first stage, followed by a subsequent loss of receptors, which translates into the observed NMDAR hypofunction at a second, later stage of disease (Olney et al. 1997; Butterfield & Pocernich 2003). Although the absolute temporal definition of these "stages" is not well defined, this theory has laid the basis for the broad but nonetheless very important concept that pathological changes affect synaptic receptor function in a different manner as pathology progresses. Similar conclusion may be drawn for glutamate accumulation. An obvious implication of this model is that therapies directed to correct NMDARs dysregulation should aim to downregulate or upregulate NMDAR activity, depending on the point of AD progression that the therapeutic intervention is happening.

Since the temporal pattern of changes to the glutamatergic system appears to be crucial to understand the molecular mechanisms underlying neurodegeneration, it may be worth considering that aberrant activation of mGluR5 may occur at a delayed phase compared to the enhancement of mAChRs. Indeed, the differential impact of P-hTau on these receptors indicates that this is a plausible idea. On the one hand, mAChRs may be first enhanced and subsequently downregulated, as previously discussed. On the other hand, and concomitantly to P-hTau accumulation, other pathological insults take place in the brain such as Aβ accumulation and glutamate excitotoxicity, which gradually spread throughout the brain and cause aberrant activation of mGluR5, among a myriad of other effects. Since glutamate accumulated at the synaptic cleft can spill-over to extrasynaptic sites (Oikonomou et al. 2012; Okubo et al. 2010; Rusakov & Kullmann 1998), where most mGluR5 is located (Conn & Pin 1997; Romano et al. 1995; Lujan et al.

1996), it is expected that glutamate present at these sites causes mGluR5 activation. In addition to glutamate, pathological activation of mGluR5 is thought to occur in the presence of Aβ (Wang 2004; Hu et al. 2014; Um et al. 2013; Hamilton et al. 2014; Renner et al. 2010). At this point when mGluR5 is overactive, it could exert enhanced inhibitory effects on mAChR. This could counterbalance the enhancement induced by P-hTau. Therefore, the overactivation of mGluR5 could potentially have two effects. First, to switch the initial upregulation of mAChR into a functional downregulation, which could result in the stoppage of compensatory changes at the level of protein expression. This, together with mechanisms of cholinergic sprouting activated in response to reduction in cholinergic activity discussed in **Chapter 4**, could explain the observed restoration of control levels of mAChRs at late stages of AD. Second, and similarly to the case of mAChRs, aberrant activation of mGluR5 could result in increased degradation or reduced synthesis of receptor as a plausible desensitisation mechanism occurring in AD (discussed in **Chapter 4**, **section 4.3**), which could account for reduced levels of mGluR5 at late stages of AD.

Future work is required to evaluate whether these speculations are true and therefore whether this is a suitable model to explain AD-associated changes in synaptic GPCRs of the glutamatergic and cholinergic families.

6.5 Relevance of tau isoforms

Although tau refers to a heterogenous group of proteins (Cleveland et al. 1977), they are encoded by a single gene that is highly conserved across species (Himmler et al. 1989; Drubin et al. 1984). Alternative splicing of tau pre-mRNA originates several tau splice variants (Drubin et al. 1984; Himmler et al. 1989; Himmler 1989; Kosik et al. 1989; Lee et al. 1988; Andreadis et al. 1992) that are translated into different protein isoforms. In the adult human brain, there are six of these isoforms whereas the number is four to five in the rat brain (Cleveland et al. 1977; Drubin et al. 1984; Francon et al. 1982; Goedert, Spillantini, Jakes, et al. 1989). In the experiments of the present thesis, transfection of rTau-shRNA is expected to knock-down the expression of all four isoforms of tau without influencing human tau (Kimura et al. 2013; Regan et al. 2015). Furthermore, co-transfecting hTau will result in the expression of one isoform, in this case full-length (2N4R) hTau, but not the others. Therefore, the finding that the phosphomimetic form of hTau 2N4R can enhance mAChRs-mediated holding current change does

not imply that other isoforms will have the same functional effects. Although this issue remains to be investigated, there are reasons to think that the variety of tau isoforms parallels differential functions in the neuronal environment.

Tau isoforms differ in the number of repeat domains (isoforms with three repeats are denoted 3R and isoforms with four, 4R) in the microtubule-binding domain and in the number of N-terminal inserts (either none, one or two) (see **Chapter 5**, **section 5.1**). These aminoacidic sequences confer each isoform distinct structural properties that may underlie their different functions. For example, 3R tau binds microtubules with a lower affinity than 4R (Butner & Kirschner 1991; Gustke et al. 1994; Goode et al. 2000; Goedert & Jakes 1990) which results in a cytoskeleton more prone to structural changes (Goode et al. 2000; Panda et al. 2003). In addition, support for separate functions of tau isoforms arises from their distinct developmental and regional/subcellular location profiles, which in turn may be isoform-dependent. Illustrating this, some isoforms are preferentially expressed in cell bodies, nucleus, dendrites or axons (Liu & Götz 2013; McMillan et al. 2008) and in specific brain regions during development (McMillan et al. 2008).

This heterogeneity of isoforms expression and function also occurs in the so-called tauopathies and specific sets of tau isoforms seem to characterise each disease (Delacourte et al. 1996; Greenberg et al. 1992; Sergeant et al. 1997; Sergeant et al. 1999; Buée & Delacourte 1999). For instance, NFTs were strongly immunostained with an antibody against 3R isoforms but not with a 4N tau antibody in brain sections of patients with severe AD (Espinoza et al. 2008; de Silva et al. 2003; Liu et al. 2001). These findings led to think that distinct tau isoforms may play different roles in neuropathological mechanisms. As an example, full-length tau and some N-terminal fragments can mediate NMDAR-induced cell death (Amadoro et al. 2006). In addition, different phosphorylated tau isoforms display different binding affinities to normal human tau, which impacts their capacity to sequester normal tau and inhibit microtubule assembly (A. D. Alonso et al. 2001). Complicating things further, it is not only the different isoform expression which may serve as a signature of disease but also the ratio between isoforms (Espinoza et al. 2008; de Silva et al. 2003; Glatz et al. 2006; Liu et al. 2001; Clark et al. 1998; Hutton et al. 1998; Spillantini et al. 1998; D'Souza et al. 1999) and their different cellular and regional expression in the diseased brain (Mori et al. 2003).

Altogether this evidence provides a solid basis to think that tau proteins exert isoform specific functions and that these may be differentially involved in AD. Accordingly, the expression of full-length hTau in this study likely does not truly mimic the pathological situation. To overcome this limitation, future work aimed to investigate the specific effects of each isoform on synaptic GPCRs activity would be required. Undoubtedly, this would provide a more comprehensive understanding of the pathological effects of tau on neuronal function.

6.6 Conclusions

Contrary to the initial hypothesis, findings from this study have shown that activation of mGluR5 does not cause synaptic impairments under the experimental conditions here. Furthermore, and in agreement with the initial hypothesis, results from this thesis support a functional interplay between mAChRs and mGluRs in the hippocampus. This could have pathological implications for AD given the specific alterations of these receptors over AD progression, which are associated with specific targeting of these receptors by one of the main neurotoxic molecules in the AD brain, P-hTau. Although the molecular substrates for this interaction remain to be investigated, this knowledge will contribute to a better understanding of synaptic dysfunction in AD and hopefully to a better design of therapeutic interventions.

The present chapter has aimed to cover the main relevant concepts and limitations of findings presented in this thesis. Currently, it is unclear whether mechanisms proposed here may account for the observations made. On a final note, it seems therefore appropriate to consider Alzheimer's own conclusions (Alzheimer 1907): *"These observations should compel us not to content ourselves with forcibly applying the knowledge we have to date to explain insufficiently understood mechanisms. Future study will enable us to gradually untangle specific mechanisms and assess their contribution to the disease."*

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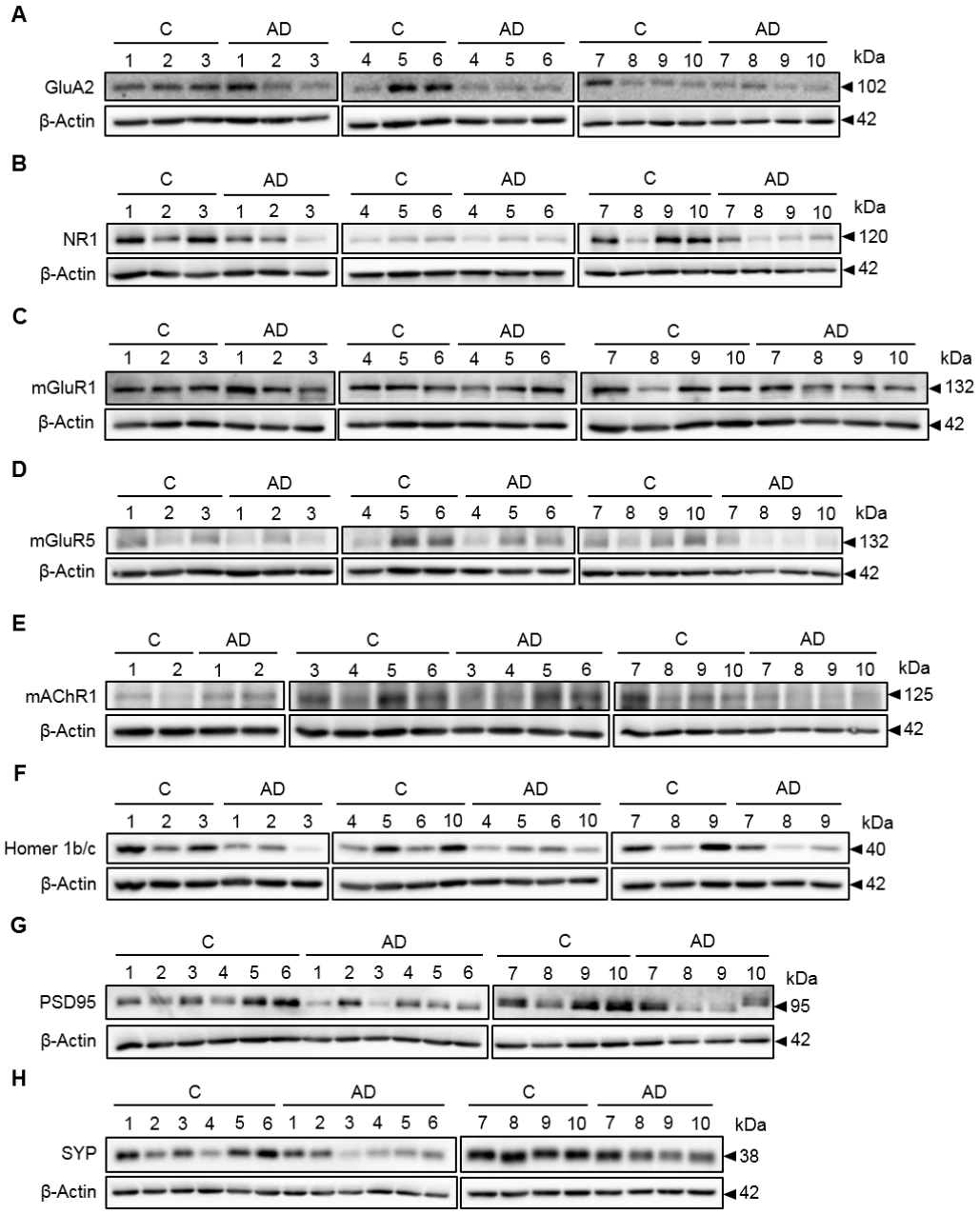
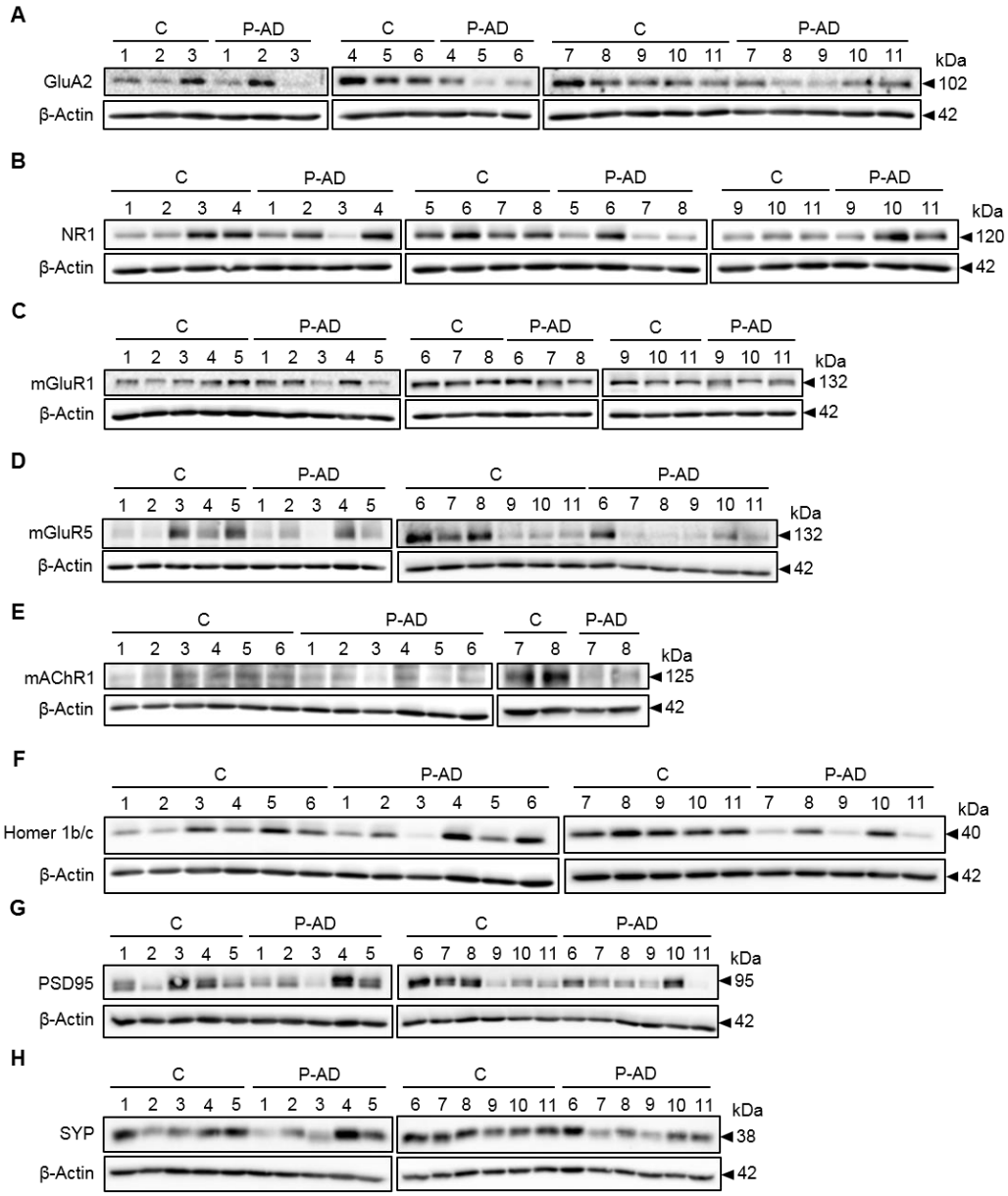
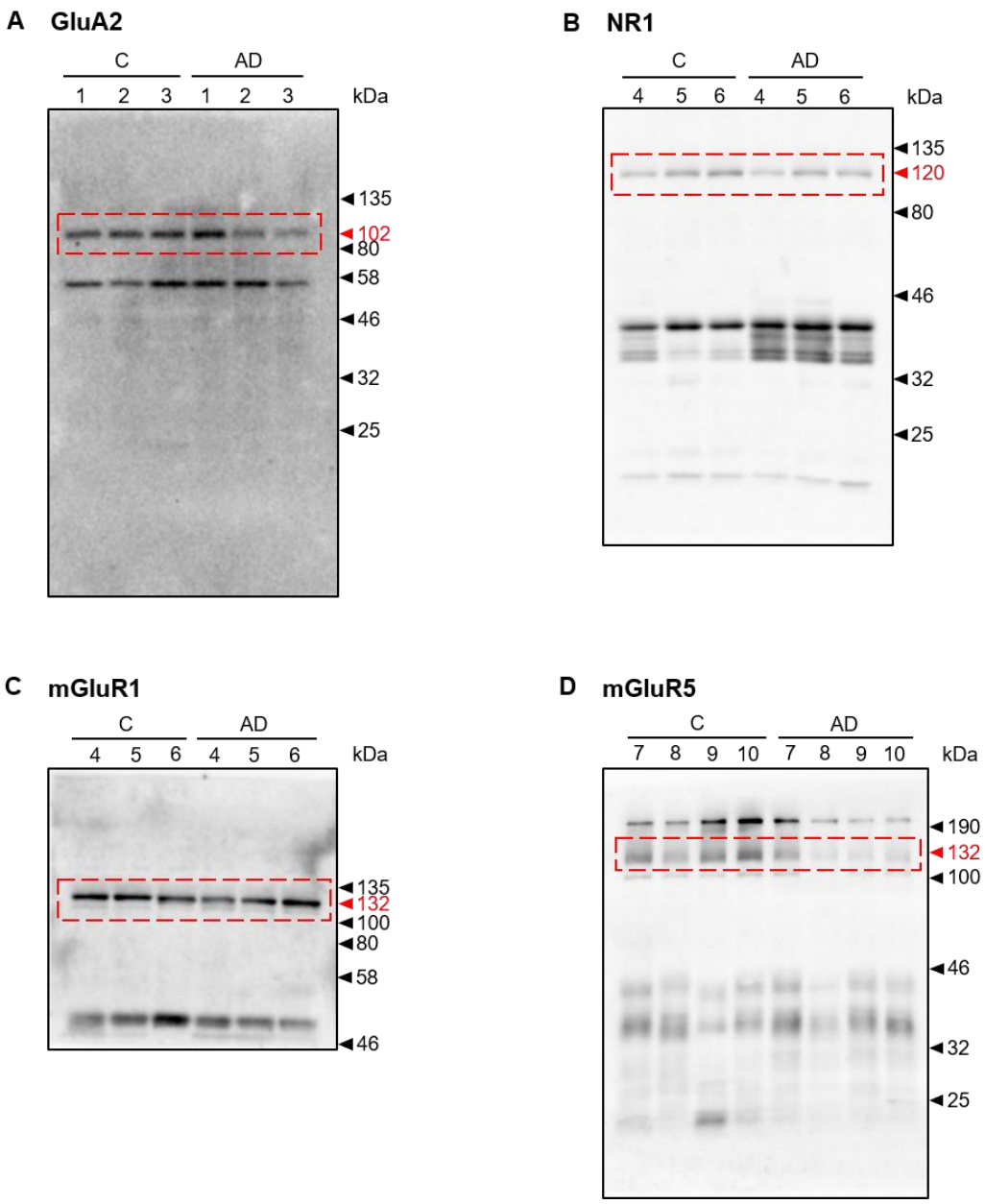
Appendix



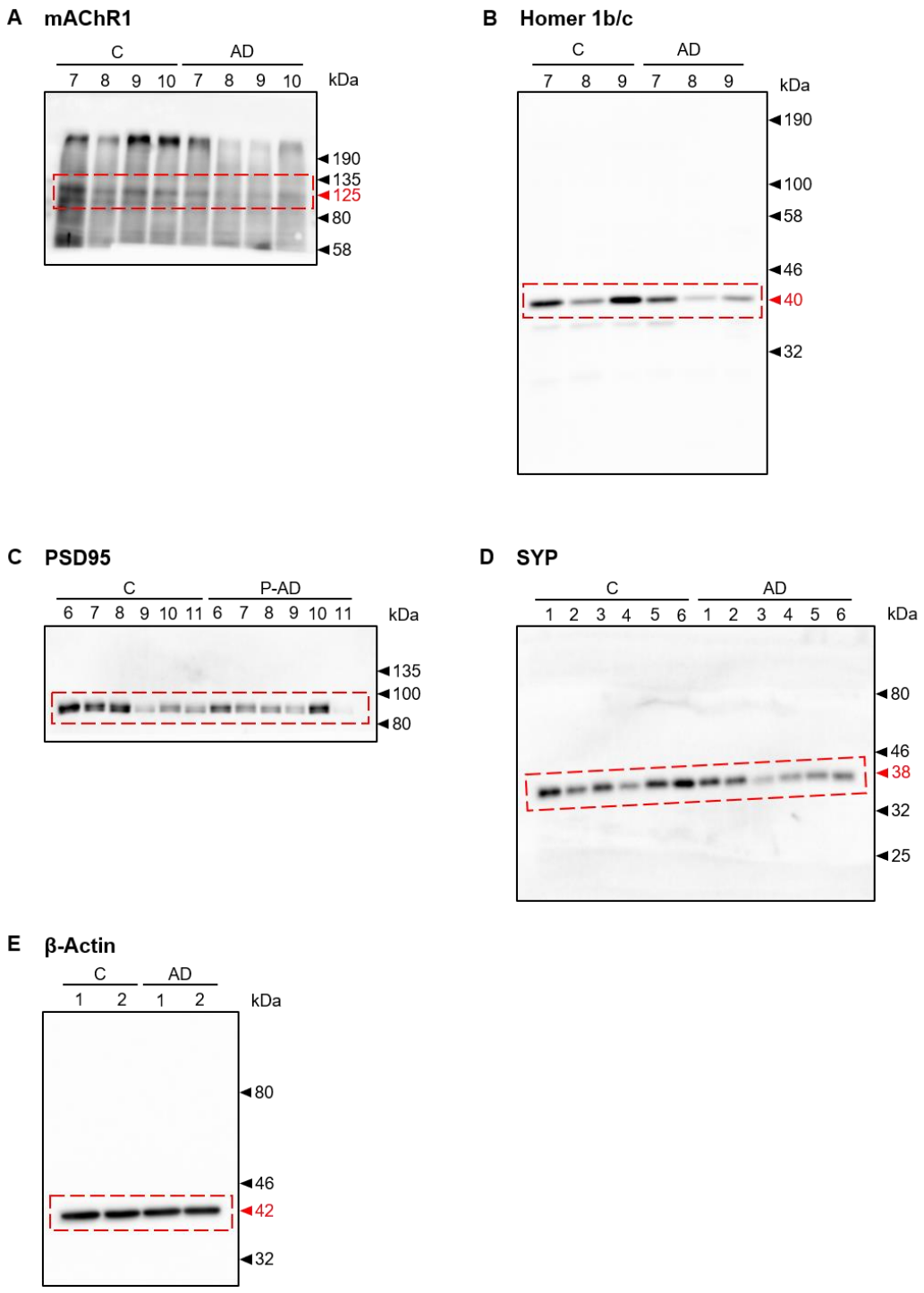
Supplementary Figure 1. Immunoblots quantified in Figures 4.2, 4.3 and 4.4. Immunoblots for **(A)** GluA2, **(B)** NR1, **(C)** mGluR1, **(D)** mGluR5, **(E)** mAChR1, **(F)** Homer 1b/c, **(G)** PSD95 and **(H)** Synaptophysin (SYP). AD: Alzheimer's disease patient, C: Non-demented aged-matched control subject.



Supplementary Figure 2. Immunoblots quantified in Figures 4.9, 4.10 and 4.11. Immunoblots for **(A)** GluA2, **(B)** NR1, **(C)** mGluR1, **(D)** mGluR5, **(E)** mAChR1, **(F)** Homer 1b/c, **(G)** PSD95 and **(H)** Synaptophysin (SYP). P-AD: Possible/probable Alzheimer's disease patient, C: Non-demented aged-matched control subject.



Supplementary Figure 3. Example of full-length immunoblots for the antibodies used in this study. (A) GluA2, **(B)** NR1, **(C)** mGluR1 and **(D)** mGluR5. Expected molecular weight of the protein of interest is shown in red. Reference molecular weights of protein marker are shown in black. Red rectangles contain the band of interest and correspond to the cropped regions shown in the main text. For technical purposes, samples were loaded in numerical order to facilitate the work, but not due to scientific reasons.



Supplementary Figure 4. Example of full-length immunoblots for the antibodies used in this study. (A) mAChR1, **(B)** Homer 1b/c, **(C)** PSD95, **(D)** SYP and **(E)** β-Actin. Expected molecular weight of the protein of interest is shown in red. Reference molecular weights of protein marker are shown in black. Red rectangles contain the band of interest and correspond to the cropped regions shown in the main text. For technical purposes, samples were loaded in numerical order to facilitate the work, but not due to scientific reasons.